TERMINATION OF GRAIN GROWTH IN CEREALS

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Declaration.

This thesis was composed by myself and describes my own original work. It has not been submitted for a degree at any other University.

CONTENTS.

ABSTRACT.

This work examines the processes taking place during grain growth and maturation in wheat which may be concerned in the termination of dry matter deposition.

Three different cultivars were used in the study and were grown under field, glasshouse and growth-room conditions. Some caryopses and detached ears were also grown in liquid culture.

Starch is a major component of dry weight, accounting for about 70% (w/w) of the endosperm. Termination of dry matter and starch deposition were shown to occur at the same point. The results suggest that reduction in assimilate supply is unlikely to lead to the cessation of dry matter deposition since sucrose levels in the endosperm were maintained beyond the stage at which starch content had reached its maximum level. Measurement of photosynthetic and respiratory activity showed that pericarp photosynthesis starts to decline before respiratory activity, and both start to fall before termination of dry matter accumulation. Photosynthetic activity paralleled changes in the chlorophyll content of the pericarp.

Comparison of starch deposition in the cultivars Fenman and Broom showed a higher final level of starch and dry weight in Fenman. This was due to higher rates of starch deposition coupled with higher rates of starch synthase activity in cv. Fenman. Affinity of starch synthase for ADPG substrate as indicated by Km was the same in both cultivars. High temperature stress reduced starch content and starch synthase activity in both cultivars, but the effects on both were greater in Fenman than in Broom.

The granule-bound form of starch synthase was found to be the most active in wheat endosperms. This activity appears to be rate-

limiting during the grain-filling period but is maintained after termination of starch deposition. However, evidence is presented that its activity may be inhibited *in vivo* towards the end of grain-filling. Although activity of ADPG pyrophosphorylase is much higher than that of starch synthase during grain-filling, it was shown to be much more sensitive to reduced water levels, induced by polyethylene glycol, when activity was greatly reduced. In this situation, starch deposition ceased. Some results indicate that onset of water loss slightly precedes termination of grain growth and starch deposition. Thus, it appears that while starch synthase activity is rate-limiting during grain-filling, reduction in ADPG pyrophosphorylase activity caused by falling water levels may be very important in determining when termination of starch deposition occurs in developing wheat endosperms.

It is suggested that termination of grain growth is the result of a programmed sequence of events which may have its origins before or very early in grain development.

ABBREVIATIONS.

(excluding standard chemical formulae).

ABA	Abscisic acid.
ADP	Adenosine diphosphate.
ADPG	Adenosine diphosphate glucose.
ATP	Adenosine triphosphate.
BSA	Bovine serum albumin.
daa	Days after anthesis.
DCMU	3-(3,4-Dichlorophenyl)-1,1-dimethylurea.
DEAE	Diethyl amino ethyl.
DHAP	Dihydroxyacetone phosphate.
DNA	Deoxyribonucleic acid.
EDTA	Ethylenediaminetetra-acetic acid.
FAO	Food and Agriculture Organisation of the United Nations.
Fru-1,6-BP	Fructose-1,6-bisphosphate,
Fru-6-P	Fructose-6-phosphate.
G-3-P	Glyceraldehyde-3-phosphate.
Glc-1-P	Glucose-1-phosphate.
Glc-6-P	Glucose-6-phosphate.
HEPES	N-2-Hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
Km	Michaelis constant.
MAFF	Ministry of Agriculture, Fisheries and Food.
MOPS	3-[N-Morpholino] propanesulphonic acid.
NAD^+	Nicotinamide adenine dinucleotide (oxidised form).

NADH	Nicotinamide adenine dinucleotide (reduced form).
NADP	Nicotinamide adenine dinucleotide phosphate.
PBD	2-Phenyl-5-(4-biphenylyl)-1,3,4-oxadiazole.
PEG	Polyethylene glycol.
PEP	Phosphoenolpyruvate.
3-PGA	3-Phosphoglyceric acid.
Pi	Inorganic phosphate.
POPOP	1,4-Di[2-(phenyloxazolyl)]-benzene.
PPi	Inorganic pyrophosphate.
RNA	Ribonucleic acid.
THO	Tritiated water.
UDP	Uridine diphosphate.
UDPG	Uridine diphosphate glucose.
UTP	Uridine triphosphate.
Vmax	Maximum velocity.

1. INTRODUCTION.

1.1 The Importance of Cereals to Agriculture.

The Gramineae, or grasses, form one of the largest plant families consisting of about 10,000 species spread throughout the world. Of these, the cereals are among the most economically important plants. A comparison of world cereal production with that of other food crops is shown in Table 1.1.

TABLE 1.1.

World Production of Major Food Types, 1984 (FAO,1984).

	(Million tonnes)
Cereals	1802
Roots & Tubers	593
Pulses	48
Vegetables, Fruits, Berries & Nuts	690

Among the cereals, rice, wheat, millet and rye form the basic diet of millions of people. Cereals and their by-products are also widely used as animal feeds. A total of 730 million hectares was devoted to cereals throughout the world in 1984 (FAO, 1984) and the figures for total production are shown in Table 1.2.

In the U.K., there are just over 4 million hectares of cereals grown, of which just under half are used for wheat production. Most of the wheat is used for milling and animal feeds (Table 1.3).

TABLE 1.2.

Cereal Production, 1984 (FAO, 1984).

	World. (Million tonnes).	U.K. (Million tonnes).
Wheat	521	14.96
Rice	470	---
Barley	172	10.96
Maize	449	---
Rye	31	0.03
Oats	43	0.55

TABLE 1.3.

Major Uses of Wheat in the U.K., 1984 (MAFF, 1985).

	(Thousand tonnes).
Milling	3621
Cereal Breakfast Foods	68
Starch and Glucose	98
Exports	1614
Seed	286
Animal Feed	5200

With the ever increasing world population, and the limited amount of land available for food production, attention has now turned to improving efficiency of land utilisation. The understanding of cereal physiology and biochemistry is essential to identify ways in which yield may be improved.

The particular objective of the work described here is to understand and identify the sequence of events leading to the termination of grain growth. Thus, the text which follows is a discussion of our present understanding of the developmental physiology and biochemistry of wheat caryopses with particular reference to events which may influence termination. For the purposes of this study, termination of grain growth has been defined as the point at which dry matter deposition ceases. In order to study this process, it is important to determine not only the events occurring at the point of termination, but also those which precede and follow it. If the causes of termination of dry matter accumulation can be identified, it may then be possible to improve yield by extending the period of grain-filling.

1.2 Developmental Morphology.

Developmental morphology is included in this introduction to familiarise the reader with the growth pattern of the species being studied and the tissues of the grain which are to be considered.

1.2.1 Development of the Wheat Plant.

The sequence of events in the growth of the wheat plant is well established (Percival, 1921; Langer, 1979).

At the onset of germination, the caryopsis absorbs water and starts to swell. The first outward indication is the appearance of the radicle, followed by two pairs of lateral rootlets. The primary shoot

elongates while protected by a colourless sheath, the coleoptile, from which the first leaf emerges.

During early development, successive leaf primordia produced by the stem apex are close together, with separation occurring subsequently through cell division in the region between adjoining primordia. As the internode grows, meristematic activity becomes restricted to the basal portion. In the leaf primordium, meristematic activity is quickly restricted to an intercalary meristem at the base. The upper part of the meristem is associated with the development of the lamina, and the lower part with the development of the sheath. Growth of the lamina within the sheaths of older leaves continues until the ligule, a membranous structure which prevents the access of foreign bodies between the leaf-sheath and stem, is differentiated. This is followed by growth of the sheath until the ligule is exposed.

Tillers arise as buds in the axils of leaf primordia at the stem apex. After germination, buds are initiated at the same rate as leaf primordia, with each bud being a replica of the parent shoot. In turn, each tiller is capable of producing secondary tillers from its axillary buds.

The main mass of root tissue is formed by adventitious roots which develop from parenchymatous tissue at the nodes just below the intercalary meristem of stem internodes. This is usually close to the ground where the internodes are short. Seminal roots arising from the root primordia present in the embryo only contribute a small amount to the total root mass.

Onset of the reproductive phase is marked by rapid elongation of the stem apex, producing leaf primordia in quick succession. Growth of the buds in the axils of these primordia gives rise to the characteristic double ridge structures of leaf and bud primordia which

indicate that the reproductive process has begun. The bud primordia then develop as spikelet primordia, with the apical dome becoming the terminal spikelet. Each spikelet primordium differentiates into florets. Maturation of the stamens and gynoaecium proceeds at the same rate and anthesis takes place soon after the emergence of the ear from the flag leaf sheath, the length of time depending on the prevailing conditions. It is characterised by the opening of the floret to expose the feathery stigmas and anthers. Dehiscence of the anthers liberates pollen, mostly within the floret. Thus, self pollination is more usual than cross-pollination.

1.2.2 Structure of the Ear and Grain.

The ear of wheat has a central rachis with spikelets positioned at nodes on either side (Fig. 1.1). The spikelets are composed of between two and nine florets joined together by a short rachilla (Fig. 1.2). Each spikelet is subtended by a pair of non-flowering glumes. Within a floret, the floral organs and later the developing grain are enclosed by a pair of flowering glumes (Fig. 1.3), the palea and lemma.

The grain (Fig. 1.4) is comprised predominantly of endosperm which is surrounded by the testa or seed coat. External to the testa is the pericarp, the fruit coat. There is a deep furrow on the ventral side which extends about halfway across the grain and is known as the crease. The embryo lies on the dorsal side at the base of the caryopsis. It is these parts which comprise the fruit or caryopsis of the wheat plant, and their morphology is described in greater detail in Section 1.2.3. In some cereals, e.g. barley, the palea and lemma adhere to the caryopsis forming a husk which is harvested with the caryopsis as part of the grain. However, this is not the case with

Fig. 1.1. Ear of Wheat

(from Hervey-Murray, 1980).

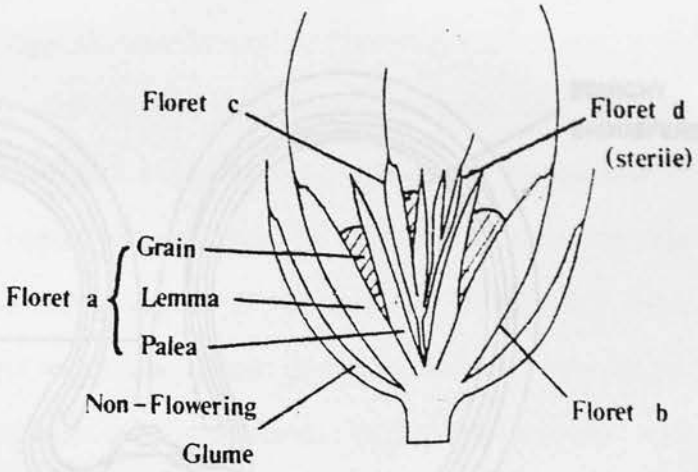


Fig. 1.2. Diagram of Spikelet

(from Bremner and Rawson, 1972).

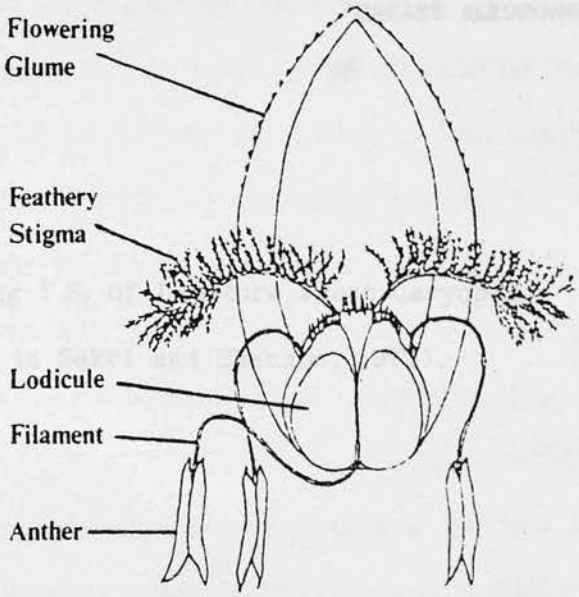


Fig. 1.3. Flower with one glume

removed (from Yampolsky, 1957).

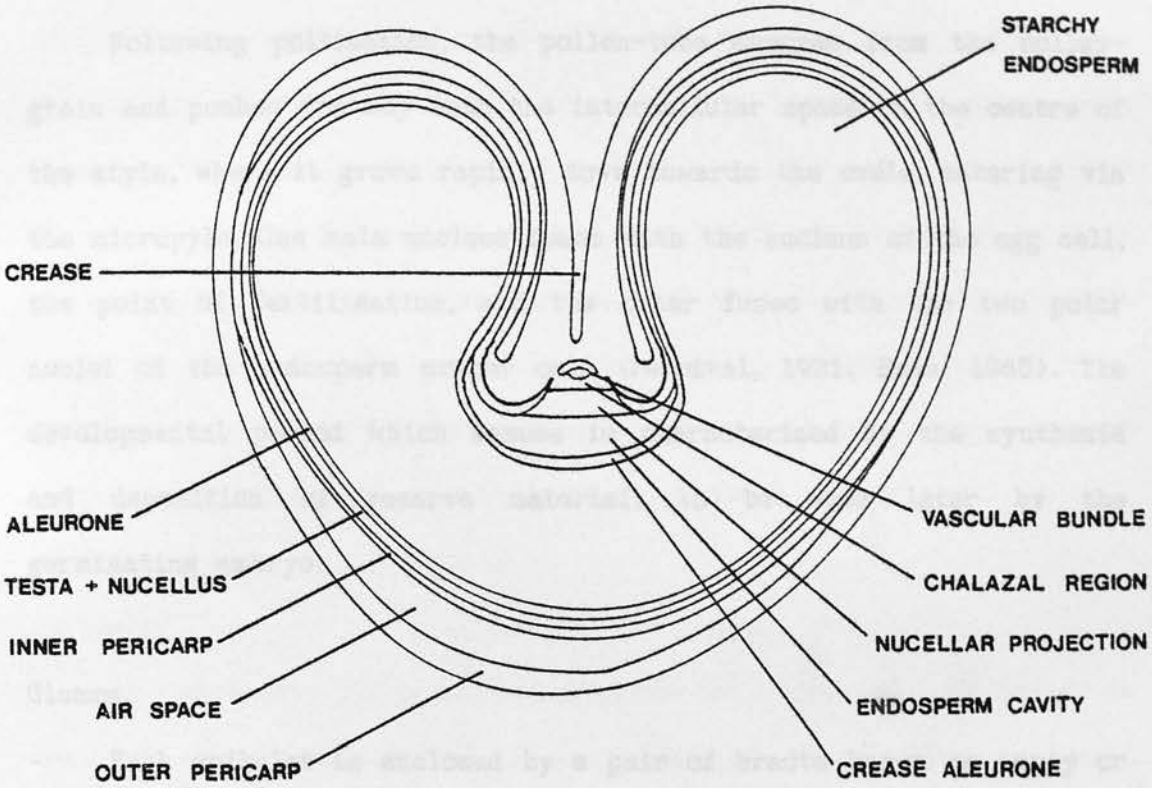


Fig. 1.4. Diagram representing T.S. of Immature Wheat Caryopsis (from a photograph in Sakri and Shannon, 1975).

wheat, so the terms grain, the part which is harvested, and caryopsis are equivalent.

1.2.3 Developmental Morphology of the Grain.

Following pollination, the pollen-tube emerges from the pollen-grain and pushes its way into the intercellular space in the centre of the style, where it grows rapidly down towards the ovule, entering via the micropyle. One male nucleus fuses with the nucleus of the egg cell, the point of fertilisation, and the other fuses with the two polar nuclei of the endosperm mother cell (Percival, 1921; Esau, 1965). The developmental period which ensues is characterised by the synthesis and deposition of reserve material, to be used later by the germinating embryo.

Glumes.

Each spikelet is enclosed by a pair of bracts known as empty or sterile glumes, and each floret by a pair of which the lower or abaxial is the lemma, and the upper or adaxial is the palea. The palea and lemma are sometimes referred to as the flowering glumes. All three types of glume are capable of photosynthesis, and together with the other green tissues of the ear are considered to make a significant contribution to grain yield (Kriedemann, 1966; Frey-Wyssling and Buttrose, 1959).

The lemma is the most photosynthetically active of the three types of glume, but its importance, particularly in comparison with the palea, decreases towards maturity (Bremner and Rawson, 1972). Consistent with the higher photosynthetic rate is an increased incidence of stomata on the lemma compared to the palea (Teare, Law

and Simmons, 1972), although Miskin and Rasmusson (1970) did not find any on the lemmae of ears of barley.

In some cultivars of wheat, the lemma may have a long, slender awn. The presence of awns in some cereals has been associated with an increase in final grain weight due to the high assimilation capacity of the awns (Vervelde, 1953; Teare, Sij, Waldren and Goltz, 1972). This is disputed by McKenzie (1972). He found that lines with awnlets were superior in yield to their awned counterparts. However, this was due to an increase in the number of ears and there was no change in final grain weight. Since all these groups used isogenic lines, the results could be attributed to genetic differences or environmental effects. Ideally, comparison of many cultivars and experiments using different environments should be used to distinguish these effects.

Pericarp.

The pericarp, which is derived from the ovary wall, is the fruit coat of the Gramineae and is composed of two layers: an outer transparent layer and an inner green one. Prior to fertilisation, the ovary wall is composed of thin walled parenchyma cells enclosed between an outer and an inner epidermis (Percival, 1921). At this stage, most of the parenchyma cells are colourless, but there are one or two layers of chlorophyll-containing parenchyma cells immediately outside the inner epidermis (Esau, 1965).

Changes in the ovary wall start with the inner epidermis. This partially disintegrates and the remaining cells elongate parallel with the long axis of the grain to form cells called tube cells. Early in development, scattered amyloplasts containing starch deposits are present, but these are lost by half way through the developmental

period when the cytoplasm of the tube cells degenerates (Morrison, 1976).

The chlorophyll-containing cells of the ovary wall elongate transversely with respect to the long axis of the grain, for which reason they are also known as cross cells. The cross cell layer is one cell thick in wheat (Percival, 1921), and two to three cells thick in barley (Cochrane and Duffus, 1979). The cells have a vacuolated cytoplasm containing many chloroplasts with abundant starch reserves and are interconnected by numerous plasmodesmata (Morrison, 1976; Cochrane and Duffus, 1979). In barley, chlorophyll levels reach a peak at 27 to 30 days after anthesis, and decline to almost zero at 40 days after anthesis (Duffus and Rosie, 1973a). The decrease in pericarp chlorophyll content is an event which may be ultimately related to termination of grain growth.

The pericarp transparent layer is formed from the colourless parenchyma cells, which become partially reabsorbed to create air spaces between the transparent and green layers. One to four layers of cells persist, and these become compressed, along with the outer epidermis, as the grain matures (Esau, 1965). In the outer epidermis of both wheat and barley, stomata have been observed among the hairs on the ventral side at the apical end (Bradbury, MacMasters and Cull, 1956; Cochrane and Duffus, 1979; Gifford and Bremner, 1981b), and there is a lipid-containing cuticular layer on the external surface (Percival, 1921; Cochrane and Duffus, 1979). Small starch grains, present in the cells of the transparent layer early in development, are gradually degraded, persisting longest in the portion covering the embryo (Percival, 1921).

Testa and Nucellus.

The testa is derived from the inner integument of the ovule. During the development of the ovule, both inner and outer integuments are composed of two cell layers, but at fertilisation the outer integument starts to disintegrate and disappears very early in grain development (Percival, 1921; Pugh, Johann and Dickson, 1932). In barley, the two layers of cells in the testa are distinct, the outer cells being smaller with denser cytoplasm and fewer vacuoles than those on the inside (Cochrane and Duffus, 1979). In the outer layer, the cells lose their contents and collapse as development proceeds, and as the grain matures, both layers become crushed into a narrow golden-brown layer (Percival, 1921; Pugh *et al.*, 1932).

Surrounding the testa on either side are two cuticular layers. The outer cuticle is derived from cuticular lamellae present between the inner and outer integuments at anthesis, and fuses with the tube cells and cross cells of the inner pericarp during development (Morrison, 1975; 1976). The inner cuticle is of nucellar origin (Morrison, 1975; Cochrane and Duffus, 1979). In barley, both cuticles have been shown to be continuous except in the crease region and at the micropyle (Cochrane and Duffus, 1983b).

The nucellus is used as a nutrient supply by the expanding endosperm and embryo in the early stages of development, with only the nucellar epidermis and a band of cells parallel to the chalazal region in the crease surviving in the later stages. The nucellar epidermis is compressed to a hyaline layer surrounding the endosperm, and produces the inner cuticle which adheres to the testa (Esau, 1965). The band of cells running parallel to the chalazal region is termed the nucellar projection, and cells with characteristics of

transfer cells have been found in this region in barley (Cochrane and Duffus, 1980).

Endosperm.

The fusion of one male gamete with the two polar nuclei produces the triploid primary endosperm nucleus. Following fertilization, the endosperm is coenocytic for a few days during which time there is a proliferation of nuclear division (Yampolsky, 1957). There is then a period when the endosperm undergoes cellularisation, usually complete by 4-5 days after anthesis (Morrison and O'Brien, 1976; Mares, Stone, Jeffery and Norstog, 1977). Morrison and O'Brien (1976) described the formation of a peripheral layer of endosperm cells by infurrowing of the wall surrounding the embryo sac, complete by two days after anthesis. Further cells were formed by radial and tangential division of the peripheral layer. However, Mares and co-workers (Mares, Norstog and Stone, 1975; Mares, Stone, Jeffery and Norstog, 1977) reported division of the endosperm into a small ventral area and a large dorsal area, which then developed separately. Cell wall formation was by freely growing cell walls along lines marked out by ingrowth of the plasma membrane.

There is a short period of cell division throughout the endosperm, after which division is confined to the outermost layer, except at the crease where there is no division (Cochrane, personal communication). Cell division in the wheat endosperm is generally considered to continue until between 14 and 20 days after anthesis (Jennings and Morton, 1963b; Evers, 1970; Briarty, Hughes and Evers, 1979; Singh and Jenner, 1982a). However, cell division in the barley endosperm has been reported as late as 30 days after anthesis (Cochrane and Duffus, 1981), and Donovan (1979) recorded increase in

DNA content of wheat grains until between 21 and 28 days after anthesis. This can only be equated with continued cell division if it is assumed that DNA per cell remained constant which was not shown. Cessation of endosperm cell division may be one step in a sequence of events leading to the termination of grain growth. Subsequent expansion of the cells is not limitless and a positive correlation between endosperm cell number and final dry weight has been demonstrated (Gleadow, Dalling and Halloran, 1982).

Differentiation of the outermost layer of endosperm cells to aleurone calls can be distinguished at 10 days after anthesis, when they appear as thin-walled, cuboidal cells with large nuclei (Morrison, Kuo and O'Brien, 1975). Thickening of the cell walls occurs by 4 weeks after anthesis according to these workers, but Evers (1970) has reported this as early as 16 days after anthesis. When comparing the results of different groups, it is important to remember that environment affects the duration of grain development and 21 days after anthesis in England is not an equivalent stage of development to 21 days after anthesis in Australia, for example. Therefore, these differences in results must be interpreted with caution. Mature aleurone cells typically have a dense cytoplasm packed with protein and phytin-containing aleurone grains surrounded by lipid droplets and numerous mitochondria (Morrison *et al.*, 1975).

Once formed, the starchy endosperm cells expand considerably and it is during this period that there is major synthesis and deposition of reserve material which will ultimately be used by the embryo in germination. Deposition of storage products is discussed in later sections. At maturity, the endosperm comprises about 80% of the final dry weight of the caryopsis and it is therefore the major component of grain yield.

Crease Region.

The crease region extends along the length of the caryopsis in the ventral furrow. Evers (1970) has suggested that the furrow is formed as a result of reduced meristematic activity in the thick-walled cells on the ventral side of the endosperm than in the opposite peripheral cells. Thus, the greater dorsal expansion causes the flanks to turn down, forming a crease.

The cross cell layer of the pericarp is composed of many cells in this region and contains a vascular bundle which is the primary, if not the sole source of nutrients of the developing caryopsis (Frazier and Appalanaidu, 1965). Two smaller vascular bundles are present at the lateral sides of the caryopsis. These are thought not to supply nutrients to the developing grain and they become distorted and gradually obliterated during development. The vascular bundle of the crease is composed of an arc of phloem elements surrounded by phloem parenchyma, with small groups of xylem vessels on the inner side. At the base of the caryopsis, the phloem elements are continuous with those of the rachilla, but at the junction of the rachilla and the caryopsis, continuity of the xylem is interrupted (Zee and O'Brien, 1970a). Maintenance of assimilate supply via the vascular bundle and crease region is essential for the continued deposition of reserve material in the endosperm. Changes in this region could regulate the uptake of assimilate. The possibility that limitation of assimilate supply may be related to termination of growth is considered in Section 1.4.

Between the vascular tissue and the endosperm cavity is the chalazal region, also known as the pigment strand, and the nucellar projection. From about two weeks after anthesis, the chalazal cells contain increasing amounts of phenolic substances, probably tannins

(Cochrane, 1983), while the cell walls contain increasing amounts of suberin and lignin (Zee and O'Brien, 1970b; Cochrane, 1983).

At about 10 to 15 days after anthesis, the nucellar projection of barley is composed mainly of thin-walled cells, except in the region bordering on the endosperm cavity. Here, some cells have acquired characteristics of transfer cells, the remainder having massively thickened cell walls (Cochrane and Duffus, 1980). Progressive degeneration of the cells bordering the endosperm cavity occurs, with the cells immediately outside them acquiring transfer cell characteristics. Only the cells in the core of the nucellar projection retain transfer cell characteristics at the end of grain filling. Cells with characteristics of transfer cells and thickened walls have also been observed in the part of the aleurone layer in the crease region, bordering on the endosperm cavity (Ayre and Angold, 1979; Cochrane and Duffus, 1980).

Embryo.

Fertilization of the egg cell, and the first divisions of the zygote occur after at least four divisions of the endosperm nucleus have taken place. The proembryo formed has a suspensor cell which is embedded in the nucellus. Development of the embryo has been described by Smart and O'Brien (1983).

The main period of embryo growth is between 15 and 30 days after anthesis. Growth is rapid, differentiation of the coleoptile, shoot, root primordia and coleorhiza are initiated, and scutellar cells become evident. The cells of the endosperm which are adjacent to the developing embryo are modified, and appear to function as a nutrient supply. They do not amass starch and protein, and they and the germ aleurone cells formed in this region appear to be progressively

degraded. The route taken by nutrients supplying the embryo is unknown and there are no plasmodesmata between the endosperm and embryo in either barley or wheat (Norstog, 1972; Smart and O'Brien, 1983). Due to the small size of the embryo, diversion of assimilates to it from the endosperm is unlikely to be significant in the termination of growth.

1.3 Yield.

If yield is to be improved, a clear understanding of the factors which affect it is essential. After ear emergence, the final yield of a wheat crop is dependent on two factors: the number of grains set per ear, and the final weight of each grain.

1.3.1 Grain Set.

The maximum number of spikelets and grains per ear is genetically determined, but the actual final number is strongly influenced by environmental conditions, particularly in the period between floral initiation and anthesis. A higher spikelet number is generally associated with longer development. Short photoperiod, which lengthens the developmental period, has been found to increase the number of spikelets formed compared to a long photoperiod (Rahman and Wilson, 1977; Pinthus and Nerson, 1984). Reduced light levels increased the duration of spikelet initiation, but also reduced the rate, giving an overall reduction in spikelet number (Rahman, Wilson and Aitken, 1977). Elevated temperature was found to delay the onset of spikelet primordia initiation and reduced the rate of primordia production and

the final number of spikelets produced (Mohapatra, Aspinall and Jenner, 1983).

The successful fertilization or otherwise of the florets is also dependent on environment. Heat stress in the meiotic phase prior to anthesis causes abnormal ovary development, which results in reduced pollen tube growth and grain set (Saini, Sedgley and Aspinall, 1983). The anthers of plants exposed to high temperatures also show abnormal development in some cases, and pollen grains were found to be shrivelled even in apparently normal anthers (Saini and Aspinall, 1982a). Saini and Aspinall (1981) found similar male sterility in plants that were water-stressed during meiosis, but female fertility was unaffected. This appears to be associated with increases in abscisic acid levels in the spikelets together with reduced grain set.

Applications of abscisic acid also reduced male fertility (Morgan, 1980; Saini and Aspinall, 1982b; Zeng, Morgan and King, 1985). Waters, Martin and Lee (1984) found that the negative effect of abscisic acid on grain set in cultured ears could be partially offset by increasing the sucrose concentration. The reduction in grain number was interpreted as abscisic acid suppressing sucrose uptake through decreases in stomatal conductance and transpiration. Higher sucrose levels in the apices of plants growing in a short photoperiod compared to a long one during spikelet initiation have also been found (Mohapatra, Aspinall and Jenner, 1982), which could give rise to the greater spikelet numbers found with short photoperiods. Fischer and Stockman (1980), who shaded plants prior to anthesis and obtained fewer numbers of competent florets due to incomplete anther development, found a temporary reduction in water-soluble carbohydrate in the ear. It is possible that this shortfall was due to decreased sucrose uptake.

1.3.2 Grain Weight.

The final weight of individual grains is thought to be dependent on three factors: the number of cells in the endosperm (Brocklehurst, 1977; Singh and Jenner, 1982b), the rate of dry matter accumulation within those cells, and the duration of dry matter accumulation (Sofield, Evans, Cook and Wardlaw, 1977). Estimates of the number of cells in the wheat endosperm vary between 60,000 and 167,000 depending on cultivar and grain position in the ear and within the spikelet (Brocklehurst, 1977; Singh and Jenner, 1982b; Gleadow, Dalling and Halloran, 1982). In barley, the number is somewhat higher, being between 200,000 and 400,000 (Cochrane and Duffus, 1981; 1983a).

Since final grain weight depends on the number of endosperm cells formed, much attention has been focussed on treatments which may increase cell number and hence final grain weight. For example, Brocklehurst, Moss and Williams (1978) caused changes in endosperm cell number by manipulation of water supply and levels of irradiance. The differences induced were due to changes in the rate rather than the duration of the cell division. Radley (1978) showed that increased grain weight was correlated with increased endosperm cell number in experiments where the grain weight was increased by removing most of the grains from the ear. Singh and Jenner (1984) found that shading reduced final grain weight and the number of endosperm cells. They also found that, with cultured ears, increasing the amount of sucrose and nitrogen in the medium led to corresponding increases in cell number and final weight. Gleadow *et al.* (1982) suggested that a higher number of cells allowed for a higher rate of dry matter accumulation. Of the six wheat lines studied, those with fewer cells also tended to have smaller cells. The number of amyloplasts within

the endosperm cells is also an important determinant of final grain weight. Evidence for this is discussed in Section 1.5.4.

The rate and duration of dry matter deposition (grain-filling) is dependent on cultivar, floret position within the ear, and the environmental conditions. Sofield, Evans, Cook and Wardlaw (1977) found that while both the duration and the rate of grain growth vary between cultivars, duration accounted for less of the difference in final grain weight. This is supported by the observations of Bingham (1967).

The position of the spikelet and the position of the floret within the spikelet both give rise to considerable differences in final grain weight. The results of Bremner and Rawson (1978) show that the spikelets with the largest grains are in the lower central portion of the ear. Within the spikelet, the *a* grain (Fig. 1.2) is usually slightly smaller than the *b* grain at maturity, and occasionally smaller than the *c* grain also. The reason for these differences is not clear and various methods have been devised to study partitioning of assimilates between the competing grain "sinks". Reducing competition for assimilate by removal of some grains shortly after anthesis, or by sterilization of florets before anthesis gives an increase in the final weight attained by the remaining grains (Rawson and Evans, 1970; Bremner and Rawson, 1978). This suggests that in the intact ear the grains are not achieving their maximum potential size. Fischer and HilleRisLambers (1978) obtained increases in weight averaging 21% with a reduction of about 80% in grain number. Reduction in the supply of assimilate by defoliation and shading experiments leads to reduced grain weights, with preferential distribution of the available assimilate to the central spikelets, and to the *b* grains (Bremner, 1972; Martinez-Carrasco and Thorne, 1979). Bremner (1972) suggested

that the faster growth rate of the *b* grain in the central spikelets is due to a higher capacity for growth rather than a favourable position in relation to the vascular system. However, distance from the source and variations in the size of the sink may also be important. When two ears containing different numbers of grains were compared, the larger sink attracted a disproportionate amount of the available assimilate (Cook and Evans 1978; 1983).

The environmental conditions prevailing during grain-filling can affect both rate and duration. Several groups of workers have studied the effect of elevated temperatures on grain-filling, and have found that while the rate of dry matter accumulation is increased, senescence is hastened and the duration reduced (Ford, Pearman and Thorne, 1976; Chowdhury and Wardlaw, 1978; Donovan, Lee, Longhurst and Martin, 1983; Nicolas, Gleadow and Dalling, 1984). To a certain extent, the increase in rate can compensate for the decrease in duration, but the higher the temperature, the greater the reduction in final weight. Asana and Williams (1965) obtained a 16% mean reduction in yield with a 6°C rise in day temperature. Although warming the ears has been observed to reduce temporarily the amount of sugars in the grain (Bhullar and Jenner, 1983), it is unlikely that limiting assimilate supply is the cause of the reduced grain weight (Chowdhury and Wardlaw, 1978; Bhullar and Jenner, 1983; Nicolas, Gleadow and Dalling, 1984). Brief periods of shading after anthesis do reduce the sucrose supply, but this is restored when the ear is returned to full illumination (Jenner, 1979; 1980a). The result of shading is a reduced rate of dry matter accumulation and final grain weight (Brocklehurst *et al.*, 1978; Jenner, 1979). Rate of dry matter accumulation is also reduced by drought, as is the duration, giving a severe reduction in final weight (Nicolas, Gleadow and Dalling, 1984; 1985).

Dry matter accumulation in the grain is largely due to the deposition of reserve material in the endosperm in the form of starch and storage protein. Synthesis of these reserves is dependent on the supply of essential precursors (assimilates) to the sites of synthesis in the endosperm. Assimilate supply and the synthesis of starch and storage protein are discussed in the following sections.

1.4 Assimilate Supply.

Carbohydrate in the grain of cereals is thought to be derived largely from photosynthesis occurring during the grain-filling period (Archbold, 1942; Thorne, 1966). Contributions from carbohydrates stored in the stem prior to anthesis may account for 5-13% of the final grain weight (Wardlaw and Porter, 1967; Austin, Edrich, Ford and Blackwell, 1977; Bidinger, Musgrave and Fischer, 1977). The major source of assimilate for the grain is from photosynthesis by the flag leaf, the stem immediately below the ear, and the ear itself (Quinlan and Sagar, 1962; Carr and Wardlaw, 1965; Thorne, 1965). About 45-50% of flag leaf assimilates are translocated to the ear (Carr and Wardlaw, 1965; King, Wardlaw and Evans, 1967), but rate of photosynthesis in the flag leaf has been shown to vary depending on demand for assimilate by the ear (King *et al.*, 1967; Evans and Rawson, 1970). The contribution of ear photosynthesis to grain filling in wheat and barley has been put between 10 and 76% (Frey-Wyssling and Buttrose, 1959; Thorne, 1965; Kriedemann, 1966). Of this, 33-42% is due to grain photosynthesis (Evans and Rawson, 1970). Morphological studies suggest that carbon dioxide can reach the green layer through stomata in the outer transparent layer (Cochrane and Duffus, 1979),

and it is possible that respired carbon dioxide from the endosperm may also be used (Duffus, Nutbeam and Scragg, 1984). Radio-labelled tracer experiments have shown that label fixed by the pericarp was later found in the endosperm and incorporated into starch (Duffus *et al.*, 1984).

Estimates of the contribution of a particular source tend to be variable as removal of one source is compensated for to a certain extent by increased contributions from the other sources (Puckeridge, 1968). Variation may also be due to increased partitioning of photosynthetic assimilates into the ear without greater photosynthesis as a result of genetic improvement of crop productivity (Gifford, Thorne, Hitz and Giaquinta, 1984).

Entry of assimilates into the grain is via the phloem which is continuous through the stem, rachis, rachilla and grain (Zee and O'Brien, 1971). The vascular bundle runs the entire length of the grain in the crease, with phloem unloading occurring along the whole length (Frazier and Appalanaidu, 1965; Sakri and Shannon, 1975).

Experiments with fluorescent tracers in wheat and barley have shown that the uptake pathway from the phloem is through the cells of the pigment strand (chalazal region) and nucellar projection to the endosperm cavity, followed by radial spread into the endosperm (Cook and Oparka, 1983). This confirmed previous predictions from anatomical studies in rice, wheat and barley (Oparka and Gates, 1981a; 1981b; Duffus and Cochrane, 1982), and is supported by the observation of transfer cells in the nucellar projection and the crease aleurone (Cochrane and Duffus, 1980). The route through the chalazal region is thought to be symplastic (Oparka and Gates, 1981b; Cochrane and Duffus, 1980; Cook and Oparka, 1983; Cochrane, 1983), with the transfer cells of the nucellar projection being the point of movement into the

apoplast. The symplastic route is intact until just prior to the final dehydration of the grain, despite the increasing amounts of phenolic substances in the chalazal cells (Cochrane, 1983). Water is thought to leave via the apoplast of the chalazal region (Oparka and Gates, 1981b), which is separated from the symplast by a layer of suberin in the later stages of development.

The pathway of assimilate movement in the endosperm is not clear. Jenner (1974b) suggested an apoplastic pathway, but the presence of plasmodesmata between maize endosperm cells (Felker and Shannon, 1980) means that a symplastic pathway cannot be ruled out. It has been suggested that restrictions on sucrose uptake might be one way in which starch deposition in the endosperm is regulated (Jenner, 1976), but experiments using radio-labelled sucrose have shown that assimilate continues to reach the endosperm until after the end of the grain-filling period (Jenner and Rathjen, 1977; Cochrane, 1985)

It is assumed that the precursors of starch and protein are sucrose and amino acids which are supplied to the developing grain in the phloem stream. Sucrose is translocated without hydrolysis (Jenner, 1974a; Donovan, Jenner, Lee and Martin, 1983; Ho and Gifford, 1984), but it appears that amino acids are metabolized during transport (Donovan, Jenner *et al.*, 1983). Sucrose is believed to be the major transport material in higher plants (Porter, 1962). Investigations into its biosynthesis have led to the conclusion that sucrose is synthesised in leaves. Triose phosphates produced by photosynthesis are translocated from the chloroplast into the cytoplasm where they are converted to fructose-6-phosphate and uridine diphosphate glucose (UDPG) (Bird, Cornelius, Keys and Whittingham, 1974). Formation of sucrose phosphate from UDPG and fructose-6-phosphate is catalysed by sucrose phosphate synthase which has been found in the leaves of both

C_3 and C_4 plants. Sucrose phosphate is hydrolysed to sucrose by sucrose phosphatase (Whittingham, Keys and Bird, 1979).

Nitrogen for grain development is thought to be derived entirely by redistribution of nitrogen from the vegetative organs, with leaves contributing 40%, glumes 23%, stem 23%, and roots 16% at 15 days after anthesis (Simpson, Lambers and Dalling, 1983). Less than 50% of the nitrogen exported by the leaves is transported directly to the ear; most is translocated to the roots where it is recycled and exported to the shoot in the transpiration stream. Due to difficulties in sampling, little is known of the assimilates present in the phloem stream in cereals. For example, the concentrations and identity of the nitrogenous compounds present are unknown. It is possible that the nitrogen is translocated in an inorganic form, as experiments with detached ear culture showed that more amino acids were present in ears cultured on ammonium nitrate than on a complete amino acid mixture (Lee, 1978). Analysis of amino acid content of parts of the ear suggests that the glumes are a significant site of synthesis of carbon skeletons for amino acids in the grain (Lee, 1978). This is supported by the observation of Simpson *et al.* (1983) that a large proportion of the nitrogen in the transpiration stream is cycled in the glumes. The presence of enzymes of amino acid metabolism in the endosperm suggests that the nitrogen supplied to the grain may not be a complete range of amino acids (Duffus and Rosie, 1978).

1.5 Starch.

Starch is the major storage product in cereal grains, accounting for 60-70% of the mature dry weight. Thus, it is a very important

determinant of final yield and a considerable amount of research has been conducted into various aspects of its deposition. As a large component of yield, termination of starch deposition is also crucial in the termination of grain growth. For these reasons, it will be discussed here in detail.

1.5.1 Structure of Starch.

The structure of starch has been recently reviewed by Manners (1985). The two main constituents are the polysaccharides amylose and amylopectin. Amylose is composed of long chains of α-(1,4)-linked α-D-glucose residues with very occasional α-(1,6)-α-D-glucosidic interchain linkages. In wheat, it comprises 26-28% of the total starch content, with an average chain length of 2100 residues (Guilbot and Mercier, 1985). Amylopectin is a highly branched structure with a molecular weight in the region of 10^7-10^8, but with only an average of 20-25 residues in an individual chain (Manners, 1985). α-(1,6)-α-D branch points account for 5-6% of the total linkages in the molecule (Guilbot and Mercier, 1985). Various models have been proposed as a possible arrangement of the constituent chains. The current theory is a "cluster" model with many of the branches running parallel to each other, which could account for the high viscosity and crystallinity of amylopectin (Guilbot and Mercier, 1985). In this model, the backbone of the molecule is composed of chains of about 60 or 45 residues, attached to which are clusters of shorter chains of about 15 residues. There are 8 to 10 times as many short chains as long ones. There have been some reports of finding material in starch which has a range of structures intermediate between amylose and amylopectin, the amount and structure varying with origin and maturity.

1.5.2 Starch Granules.

Almost all the starch in cereal grains is located in the endosperm where it is deposited as starch granules within amyloplasts.

There are two populations of starch granules, designated type A and type B (May and Buttrose, 1959). A-type amyloplasts, which contain the A-type granules, are first observed two to three days after anthesis. Buttrose (1963) and Parker (1985) described each A-type granule as enclosed in a separate amyloplast double membrane, but Williams and Duffus (1978) found many small granules within the amyloplast and suggested that starch is initiated at several sites which then coalesce to form a single granule. Others reported the formation of lipid structures into one or more sacs in the stroma of the amyloplast which is succeeded by an accumulation of particulate material in association with the sacs. This material chemically resembles starch and, when a critical quantity has accumulated, it crystallizes into the nucleus of a granule (Jenner, 1982a; Banks, Greenwood and Muir, 1974). Evers (1971) proposed that the starch granule is built up by deposition of starch around a minute spherical "nucleus".

The second population of B-type granules appears at around 14 days after anthesis, arising in the stroma of the A-type amyloplasts, and in protusions of the amyloplast membrane (Buttrose, 1960; Parker, 1985). Whether the B-type granules were actually released to form separate B-type amyloplasts was questioned by Parker (1985).

The number of initiation sites for starch synthesis and the number of starch granules formed may be important determinants of final yield. It is not known what triggers initiation of starch synthesis. Also unknown is whether or not starch granules completely fill the amyloplasts. If the amyloplasts are filled and the membrane

restricts growth beyond a certain maximum size, the size and number of amyloplasts may be critical in determining final yield and when termination of starch deposition occurs.

The A-type granules are large and lenticular, ranging in size from 10-36μm (Dengate and Meredith, 1984), or 15-40μm (Evers, 1971). The spherical or polygonal B-type granules are less than 10μm in size. They are much more numerous than the A-type, accounting for 97% of the total granule population although they only contribute one third of the final starch content (Evers, 1973; Hughes and Briarty, 1976; Evers and Lindley, 1977). The starch granules contain about 2% non-carbohydrate material, including about 0.5% protein and about 1% lipid (Guilbot and Mercier, 1985). Some of this lipid is classified as "internal". These are exclusively monoacyl lipids which may occur as inclusion complexes within amylose helices, or which may be tightly entrapped in spaces between amylose and amylopectin molecules (Morrison, 1981).

The arrangement of amylose and amylopectin in the starch granule is not fully resolved and has been discussed in some detail by Banks and Muir (1980). X-ray diffraction of starch granules gives a pattern indicative of crystalline order. The amylose, in cereals sometimes complexed with lipid, apparently exists in a helical form, but without the three-dimensional order necessary to give the crystalline diffraction pattern. This implies that amylopectin is the principal crystalline component. Studies with different varieties of maize containing varying amounts of amylose and amylopectin support this. Optical evidence is consistent with amylopectin molecules being radially aligned within the granule, and studies with tritium-labelled starch granules suggest that the non-reducing ends of the molecules are orientated towards the granule surface (Nordin, Moser, Rao, Giri

and Liang, 1970). Electron microscopy of a starch granule showed an alternating ring system which appear light and dark in silver-fixed preparations. The lighter, less silver-reactive rings correspond to the crystalline shells, and the darker rings to the more amorphous shells. It is thought that the crystalline regions are associated with the "clusters" of the amylopectin molecules (Guilbot and Mercier, 1985).

1.5.3 Starch Biosynthesis.

The deposition of starch in the endosperm parallels the increase in dry weight during development (Jennings and Morton, 1963a; Cerning and Guilbot, 1973). There is a steady increase in starch content from about 10 days after anthesis, and this levels off at the stage at which the grain water content starts to drop. At maturity, starch accounts for most of the endosperm dry weight (Jennings and Morton, 1963a).

Synthesis of starch is thought to be from sucrose. However, no-one has been able to produce a time-course of labelled intermediates linking sucrose to starch and most evidence is indirect, based on enzyme activities. Jenner (1968a) showed that starch synthesis continued for several days in detached ears cultured on sucrose solutions, but there was no further starch synthesis after 24 hours in ears cultured on water. Further experiments with carbon-14 labelled sucrose showed incorporation of the label into starch (Jenner, 1973; Jenner and Rathjen, 1975). Experiments with the label equally in the glucose and fructose moieties, or in the fructose moiety exclusively, showed that both are converted to starch and at approximately equal rates (Jenner, 1973). Most labelled sucrose was converted to starch at 28 days after anthesis (Jenner and Rathjen, 1975), although the

capacity of the endosperm to absorb sucrose increased throughout development (Jenner and Rathjen, 1975; 1977). These experiments did not distinguish between label derived directly from sucrose and label which was derived by inversion of sucrose, metabolism, possibly via glycolysis, and reconversion into intermediates of starch synthesis.

The possibility that sucrose is hydrolysed prior to entry into the endosperm cells has been investigated. Sakri and Shannon (1975) observed high levels of labelled monosaccharides in the pericarp and endosperm shortly after exposure of the flag leaf to $^{14}CO_2$ and suggested that hydrolysis might be a prerequisite for the translocation of sucrose from the phloem to the endosperm, although not all their results supported this theory. Jenner (1974a), working with whole grains, found that extracellular acid invertase was capable of hydrolysing sucrose at a rate sufficient to support the rate of starch synthesis observed. However, inhibition of the hydrolysis had no effect on the uptake of sucrose or the speed of its conversion to starch. He concluded that absorption of sucrose is not dependent on extracellular hydrolysis. Chevalier and Lingle (1983) later identified most of the invertase activity as being associated with the pericarp, whereas sucrose synthase activity was associated with the endosperm. These workers also studied the sucrose, glucose and fructose composition of leachable and non-leachable fractions of grain tissues, finding that sucrose levels were high and that glucose and fructose levels were low in both fractions of all tissues. The sucrose content of the leachable fraction of the endosperm was found to increase as the sucrose synthase activity declined. Rijven and Gifford (1983) found that starch synthesis was fastest in endosperm slices if they were incubated on a sucrose medium rather than glucose and/or fructose. Thus, the weight of the evidence supports the proposition

that hydrolysis of sucrose is not required for uptake into endosperm cells. Rijven and Gifford (1983) provide some evidence for an active transport mechanism of proton-sucrose co-transport concurrent with diffusional influx and efflux.

Various pathways have been proposed for the synthesis of starch from sucrose, once the sucrose has entered the endosperm cell. A possible route for the conversion is shown in Fig. 1.5 (Duffus, 1984), taking into account present knowledge of enzyme activity and likely properties of the amyloplast double membrane.

Initial cleavage of sucrose could be by invertase or by sucrose synthase (sucrose-UDP (ADP) glucosyl transferase). It seems likely that sucrose synthase is the principal enzyme involved as high levels of activity have been found in the endosperm of both wheat and barley (Baxter and Duffus, 1973a; Meredith and Jenkins, 1976; Chevalier and Lingle, 1983), whereas invertase activity in the endosperm is low (Kumar and Singh, 1980; Chevalier and Lingle, 1983). This pathway has the advantage of conserving the energy of the glycosidic bond which would be lost by invertase action (Rijven and Gifford, 1983). Sucrose synthase is capable of functioning with either adenosine diphosphate (ADP) or uridine diphosphate (UDP) as the glucose acceptor forming adenosine diphosphate glucose (ADPG) or uridine diphosphate glucose (UDPG). Baxter and Duffus (1973a) showed that the enzyme from barley had an activity up to three times as great with UDP than with ADP, and kinetic data from the rice enzyme show the Michaelis constant (Km) with ADP to be considerably higher than the Km with UDP (Murata, Sugiyama, Minamikawa and Akazawa, 1966; Nomura and Akazawa, 1973).

The route taken following the sucrose synthase reaction is unclear. Since the permeability properties of the amyloplast membrane are largely unknown, it has not been possible to put forward a

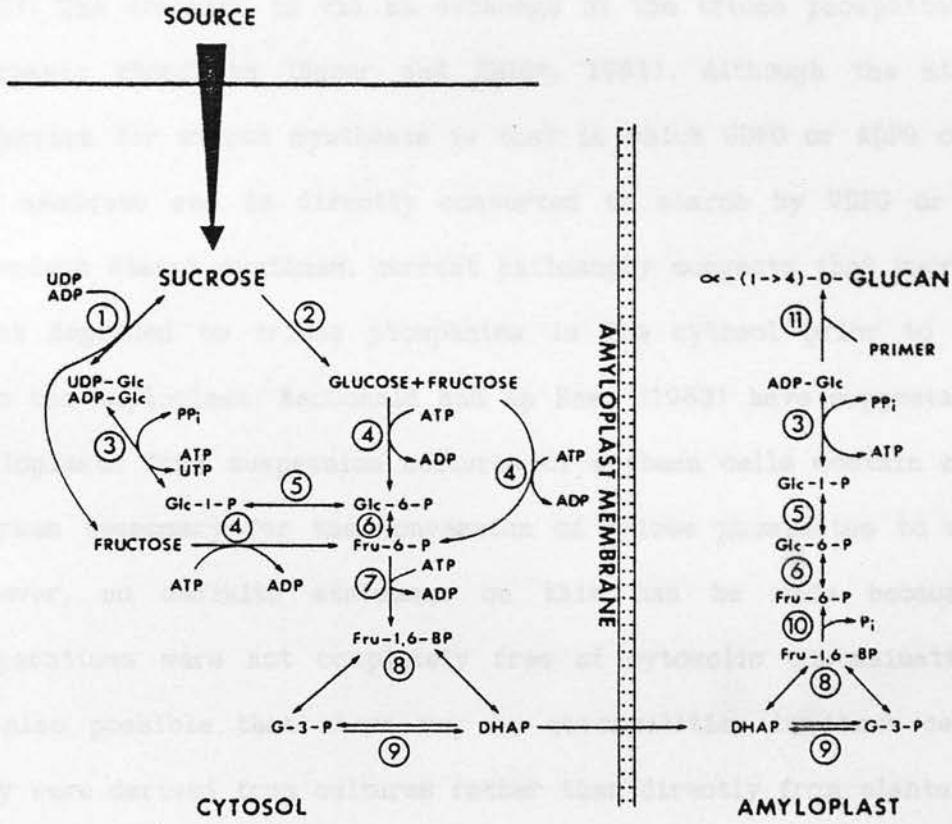


Fig. 1.5. Scheme for the Conversion of Sucrose to α-(1→4)-D-Glucan.

1-sucrose synthase; 2-invertase; 3-ADPG and UDPG pyrophosphorylases; 4-hexokinase; 5-phosphoglucomutase; 6-phosphohexoisomerase; 7-phosphofructokinase; 8-aldolase; 9-triosephosphate isomerase; 10-fructose-1,6-bisphosphatase; 11-starch synthase.

generally accepted pathway for the conversion of sucrose to starch. It may be the case that, like the chloroplast membrane, the amyloplast membrane is significantly permeable only to the triose phosphates and 3-phosphoglyceric acid (3-PGA) (Fliege, Fluggee, Werdan and Heldt, 1978). The transfer is via an exchange of the triose phosphates with inorganic phosphate (Heber and Heldt, 1981). Although the simplest mechanism for starch synthesis is that in which UDPG or ADPG crosses the membrane and is directly converted to starch by UDPG or ADPG-dependent starch synthase, current philosophy suggests that sucrose is first degraded to triose phosphates in the cytosol prior to uptake into the amyloplast. MacDonald and ap Rees (1983) have suggested that amyloplasts from suspension cultures of soybean cells contain all the enzymes necessary for the conversion of triose phosphates to starch. However, no definite statement on this can be made because the preparations were not completely free of cytosolic contamination. It is also possible that there may be abnormalities in these cells as they were derived from cultures rather than directly from plants.

After conversion of triose phosphate back to glucose-1-phosphate (Fig. 1.5), the synthesis of starch is thought to be catalysed by the enzymes ADPG (UDPG) pyrophosphorylase (ATP (UTP): α-D-glucose-1-phosphate adenylyl transferase), starch synthase (ADPG (UDPG) α-(1,4)-glucan α-4-glucosyl transferase) and branching or Q enzyme (α-(1,4)-glucan, α-(1,4)-glucan-6-glycosyl transferase). Activity of these enzymes is likely to be very important in determining the amount of starch deposited and therefore the final yield. Maintenance of their activity may be a crucial factor affecting when termination of starch deposition occurs.

ADPG Pyrophosphorylase.

Both ADPG and UDPG can be formed by pyrophosphorylase reactions in which ATP or UTP combines with glucose-1-phosphate to form ADPG or UDPG and pyrophosphate (Espada, 1962). Alternatively, sucrose synthase also generates these nucleotide sugars. However, as previously mentioned, sucrose synthase is most active with UTP forming UDPG, whereas the starch synthesising mechanism exhibits greatest activity with ADPG. Analysis of ADPG pyrophosphorylase activity in cereal grains such as wheat (Turner, 1969; Kumar and Singh, 1983) and barley (Baxter and Duffus, 1973a) shows an increase during starch synthesis and a decline to very low levels when starch synthesis ceases. In these measurements, it is assumed that the activities recorded *in vitro* are equivalent to those *in vivo* and this applies to all other enzyme activities reported. Evidence supporting the role of ADPG pyrophosphorylase in starch synthesis comes from work with a maize mutant which lacks ADPG pyrophosphorylase and in which the starch levels are about 25% of the normal (Tsai and Nelson, 1966). These results suggest that at least 75% of starch is synthesised through the ADPG pathway. However, there may be some lesion other than that affecting the ADPG pyrophosphorylase activity which is limiting the amount of starch synthesised, so the validity of this conclusion is open to question.

Studies on ADPG pyrophosphorylase from leaves have shown that it is subject to allosteric regulation. It is activated by 3-PGA and to a lesser extent by other glycolytic intermediates such as phosphoenolpyruvate (PEP), fructose-6-phosphate and fructose-1,6-bisphosphate, and inhibited by inorganic phosphate (Pi) and ADP (Ghosh and Preiss, 1966; Sanwal, Greenberg, Hardie, Cameron and Preiss, 1968). The inhibitory effect of phosphate can be partially overcome by

increasing the 3-PGA levels. 3-PGA is the primary CO_2 fixation product in photosynthesis. It has been postulated that starch synthesis during photosynthesis is stimulated by activation of ADPG synthesis via increases of 3-PGA concentration in the chloroplast and a concomitant decrease in inorganic phosphate due to photophosphorylation. At night, the absence of photosynthesis leads to an increase in the phosphate levels, and starch degradation ensues (Preiss, MacDonald, Singh, Robinson and McNamara, 1985). Studies of isolated chloroplasts have shown that changes in 3-PGA and Pi concentrations can be correlated with observed changes in rate of starch synthesis in this way (Heldt, Chou, Maronde, Herold, Stankovic, Walker, Kraminer, Kirk and Heber, 1977; Peavy, Steup and Gibbs, 1977; Portis, 1982). Recent results have shown that ADPG pyrophosphorylase from spinach leaves is also inhibited by pyrophosphate (Preiss *et al.*, 1985).

The pyrophosphorylase from maize endosperm is the most studied of those from non-photosynthetic tissues (e.g. Vidra and Loerch, 1968; Dickinson and Preiss, 1969a; 1969b; Hannah and Nelson, 1975). Activation by 3-PGA occurs but to a much smaller extent than for the leaf enzyme, and the same is true of inhibition by phosphate. Inhibition of the sweetcorn enzyme by pyrophosphate has also been found (Amir and Cherry, 1972). It has been suggested that the lesser sensitivity of the maize enzyme to allosteric regulation may reflect differences between endosperm and leaf cells with respect to intracellular levels of metabolites. It was also suggested that non-photosynthetic tissue had no need for allosteric regulation (Preiss and Levi, 1980). That is, as starch turnover is much less, being effectively a system specialising in starch synthesis and net deposition, it may be that the pyrophosphorylase has evolved to a form which is comparatively insensitive to activation and inhibition.

This assumes that the pyrophosphorylase from leaves and reserve tissues is the same protein which has not been shown. The regulatory mechanisms controlling the ADPG pyrophosphorylase in the endosperm may then be completely different.

One theory is that an important aspect controlling starch biosynthesis in the endosperm of several types of cereal may be the regulation of synthesis of the starch biosynthetic enzymes (Preiss and Levi, 1980; Preiss et al., 1985). However, since the data referred to does not distinguish change in overall activity due to differences in the amount of enzyme protein present from that due to activation or inhibition of specific activity , it is not really possible to say whether this is so or not.

Starch Synthase.

Prior to 1961, it was thought that the formation of α-(1,4)-glucosidic linkages in plants was catalysed by phosphorylase (Hanes, 1940). However, it is now generally accepted that starch synthase catalyses the synthesis of most, if not all, starch α-(1,4)-glucosidic linkages. The basic requirements for starch synthase activity are the sugar-nucleotide donor and a primer. The primer requirement can be satisfied by either amylose, amylopectin, glycogen, starch granules, or various oligosaccharides of the maltodextrin series (Ghosh and Preiss, 1965; Cardini and Frydman, 1966). The presence of starch synthase was inferred from studies with glycogen synthase (de Fekete, Leloir and Cardini, 1960) and was shown to incorporate the glucose moiety of UDPG into a primer chain of α-(1,4)-linked residues, with release of UDP (Leloir, de Fekete and Cardini, 1961). It was subsequently found that ADPG was a better substrate than UDPG in terms of rate of reaction and amount incorporated into starch in a variety of plant

tissues (Recondo and Leloir, 1961; Murata and Akazawa, 1964; Tanaka and Akazawa, 1968; Baxter and Duffus, 1971). Since the starch synthases of leaves appear to be specific for ADPG (Murata and Akazawa, 1964; Ghosh and Preiss, 1965), it is likely that the ADPG pathway is predominant in plant tissues generally. Even in storage tissue where starch synthases can use both ADPG and UDPG, the Km for UDPG is many times higher than for ADPG, and the rate of incorporation of UDPG is usually one third to one tenth of that observed with ADPG (Cardini and Frydman, 1966; Murata, Sugiyama and Akazawa, 1964). Other nucleotide-glucosyl donors have been demonstrated to work in several tissues, but in all cases ADPG was superior (Frydman and Cardini, 1967).

Starch synthase exists in two forms, one which is bound to the starch granules and a second which is soluble (Frydman and Cardini, 1964). The specificity of the soluble enzyme for a particular glucosyl nucleotide is sometimes different from that of the bound enzyme (Ghosh and Preiss, 1965; Frydman and Cardini, 1966; Baxter and Duffus, 1973b). It has been suggested that these two forms are the same enzyme and that adsorption or entrapment in the starch granule may change the properties with respect to the glucosyl donor. Frydman and Cardini (1967) showed that mechanical disruption could change the glucosyl donor specificity of the granule-bound enzyme. Similarly, MacDonald and Preiss (1983) found that solubilization of the bound enzyme from maize kernels by grinding and digesting with starch-degrading enzymes meant that the enzyme could no longer utilise UDPG as a glucosyl donor. In certain mutants of maize where the granule is devoid of amylose, the bound form of starch synthase is not present (Nelson and Rines, 1962; Frydman, 1963), although Murata, Sugiyama and Akazawa (1965) have found soluble starch synthase activity in such

grains of rice. It has been shown that amylose is capable of adsorbing the soluble enzyme, transforming it into a particulate enzyme (Akazawa and Murata, 1965). Thus, it appears that the adsorption of the synthase to the granule may be due to the presence of amylose. However, since the precise nature of the lesion in the mutant rice is not fully described, it may be that the two enzymes are distinct and that the mutations which give rise to varieties without amylose also cause the absence of the bound form of starch synthase.

The soluble starch synthase from a variety of sources has been shown to exist in multiple forms (Ozbun, Hawker and Preiss, 1971; 1972; Hawker and Downton, 1974; Pisigan and del Rosario, 1976; Kreis, 1980). The number of isoenzymes found is between two and four, with most of the activity in two peaks. In most cases, one of the fractions is able to catalyse the formation of α-(1,4)-linkages without addition of a starch or oligosaccharide primer. Solubilized granule-bound starch synthase of maize kernels has also been resolved into two fractions, one of which shows unprimed activity (MacDonald and Preiss, 1983).

Branching Enzyme.

Branching enzyme activity, which catalyses the formation of α-(1,6)-linkages, is associated with starch synthase activity, but very little is known about its mechanism of action and the nature of the glucan substrate. The enzyme from potato tubers has been purified and studied in detail (Borovsky, Smith and Whelan, 1975a) and can utilize amylose chains of about 40 glucose units length or more as substrates, although this varies with experimental conditions (Borovsky, Smith and Whelan, 1975b; 1976). From experiments using radioactive labels in which the enzyme transferred the label between amylose chains,

Borovsky *et al.* (1976) proposed two possible models for the catalytic mechanism. In the first, the enzyme forms a covalent bond with a donor amylose chain and this complex interacts with a second acceptor amylose chain to form the α-(1,6)-linkage. In the second, two amylose chains form a double helix which facilitates the enzyme action by transferring a portion of one chain to the other forming the linkage.

Branching enzyme from spinach leaves has been resolved into two fractions (Hawker, Ozbun, Ozaki, Greenberg and Preiss, 1974), one of which is closely associated with starch synthase activity. The properties of the two fractions were found to be very similar, and both stimulated the unprimed activity of one of the soluble starch synthase fractions described previously. Multiple fractions of branching enzyme activity from maize endosperm have also been found (Boyer and Preiss, 1978; 1981). These fractions have been shown to be immunologically distinct although the amino acid composition shows that they are related (Fisher and Boyer, 1983).

Phosphorylase.

Whether phosphorylase is active in the biosynthetic pathway or not remains unclear. It catalyses the transfer of glucose residues from glucose-1-phosphate to a glucosyl chain with the release of inorganic phosphate (Hanes, 1940). Estimates of the Km of phosphorylase for glucose-1-phosphate range from 1 to 12.5mM in different plant tissues (Alexander, 1973; Frydman and Slabnik, 1973; Burr and Nelson, 1975; Chen and Whistler, 1976) which are much higher than the calculated concentrations of glucose-1-phosphate present (Preiss and Levi, 1980). These figures are also greater than the Km of ADPG pyrophosphorylase for glucose-1-phosphate of 0.2mM in sweet corn (Amir and Cherry, 1972). Therefore, it seems likely that most of the

available glucose-1-phosphate will be used by the pyrophosphorylase, thus preventing any significant participation of phosphorylase in starch synthesis. The ADPG formed is also able to inhibit phosphorylase activity (Burr and Nelson, 1975).

Phosphorylase activity has been found in maize (Tsai, Salamini and Nelson, 1970; Ozbun, Hawker, Greenberg, Lammel, Preiss and Lee, 1973) and in barley (Baxter and Duffus, 1973c) at all stages of development. In very young barley endosperm, there is unprimed phosphorylase activity which may be present to synthesise the primers required for starch synthase (Baxter and Duffus, 1973c). Sastry, Sundaresan and Pande (1979) found that barley mutants with reduced starch content had high phosphorylase levels and suggested that phosphorylase had a degradative role. Again, however, the results with mutants have to be interpreted with some caution as the full nature of the lesion is unknown.

The presence of activity of other starch hydrolysing enzymes in the developing barley endosperm has also been reported (Duffus and Rosie, 1973b). α-Amylase activity, like that of phosphorylase, peaked between 25 and 30 days after anthesis. Latent β-amylase reached a constant maximum level at 35 days after anthesis, although available β-amylase peaked at 25 days after anthesis and had declined to zero by 45 days after anthesis. β-Amylase was the most active of the three enzymes. The role of these enzymes is not known, but it is possible that some starch turnover is taking place during development.

1.5.4 Effect of Environment on Starch Deposition.

The effects of elevated temperatures on starch deposition have been extensively studied in cereals. As previously mentioned, the final

yield per grain is reduced at higher temperatures due to a reduction in the duration of grain-filling which is not compensated for by the increase in the rate of dry matter deposition (Sofield, Evans, Cook and Wardlaw, 1977). It has been shown that the reduction in grain weight observed is accompanied by a parallel decrease in the starch content of the endosperm (Spiertz, 1977). This is largely due to a marked reduction in the number of B-type starch granules in the endosperm (Hoshikawa, 1962; Bhullar and Jenner, 1985; MacLeod, 1986). Most of these workers found no significant change in the number of A-type granules, but MacLeod (1986) found some evidence to suggest that these too were reduced in number in barley, although those remaining were of a larger average diameter. It is unlikely that these reductions are due to a decrease in the supply of sucrose as levels in the rachis are higher at higher temperatures (Bhullar and Jenner, 1983). Bhullar and Jenner (1983) suggested that uptake of assimilate into the endosperm might be depressed as they had found reduced sucrose levels, but MacLeod (1986) found that the sucrose level in barley endosperm was unaffected by increased temperatures, as did Jones, Ouattar and Crookston (1984) with maize.

These changes are presumably brought about by changes in temperature-dependent metabolic events such as enzyme activity. Thus, it has been shown that the activity per endosperm of UDP-sucrose synthase in barley (MacLeod, 1986), and the specific activities of phosphorylase, branching enzyme and both forms of starch synthase from potatoes (Mangat and Badenhuizen, 1971) are reduced at temperatures of 30°C. Although ADPG pyrophosphorylase activity per kernel in maize is unaffected by temperatures of up to 25°C (Ou-Lee and Setter, 1985), these authors did not investigate the effect of higher temperatures on enzyme activity.

Other environmental effects on starch deposition have not been studied in as much detail as temperature. In drought conditions, the water content of the grain is hardly affected, while the water content of leaves and stems is severely reduced (Wardlaw, 1971). Mild water stress had no effect on starch deposition, but a severe stress reduced the rate of starch accumulation (Brocklehurst *et al.*, 1978). The duration of grain-filling has been reported as either unaffected (Brocklehurst *et al.*, 1978) or reduced (Wardlaw, 1971; Nicolas *et al.*, 1984; 1985) by water stress. The number of starch granules in the endosperm was reduced, and this was attributed to fewer B-type granules (Brocklehurst *et al.*, 1978; Nicolas *et al.*, 1984; 1985). Light is not itself required for starch accumulation provided there is ample substrate available (Jenner, 1968a), but shading or low irradiance during grain-filling does reduce the amount of starch deposited and this is attributed to a reduction in the assimilate supply (Sofield, Evans, Cook and Wardlaw, 1977; Brocklehurst *et al.*, 1978). However, it has also been shown that the activity of starch synthase from plants shaded during grain-filling is reduced (Mengel and Judel, 1981). Thus, it would appear that environment can affect the synthesis and deposition of starch in many different ways, as a result of which there can be large differences in final grain weight.

1.6 Storage Proteins.

One of the reasons for the very high demand for wheat compared to other cereals is that it, and to a lesser extent rye, gives a flour that produces an elastic, cohesive dough which can be used in the

manufacture of leavened bread. The unique cohesive properties of wheat dough are due to the proteins it contains (Wall, 1979).

Although considerably less significant than starch, proteins contribute an average of 12-13% of the dry weight of wheat grains. Most of this is found in the endosperm, with a small amount in the embryo (Duffus and Slaughter, 1980). The endosperm proteins can be divided into two groups: the cytoplasmic proteins, and the storage "gluten" proteins. There are two types of storage protein in wheat: glutenins and gliadins. The boundary between the two groups is not distinct. Of the total protein content of the grain, 40-50% are gliadins and 30-40% are glutenins (Rhodes and Jenkins, 1978). All the proteins are synthesised throughout development (Graham and Morton, 1963), but varieties differ in their polypeptide composition (Graham, 1963).

Total nitrogen in the wheat grain follows the pattern of dry weight accumulation (Feller, 1978; Gleadow *et al.*, 1982). However, there is evidence that synthesis of nitrogen-containing compounds, of which proteins are predominant, may continue slightly after cessation of dry matter accumulation (Barlow, Lee and Vesk, 1974). This suggests that termination of deposition of factors contributing to final dry weight may not all occur at the same point.

Although nitrogen-containing compounds contribute to final dry weight, there is no correlation between final yield and amount of nitrogen present in the grain when different cultivars are compared (Donovan, 1979). Donovan, Lee and Hill (1977) found that a higher protein content was associated with a higher protein synthetic capacity and hence a higher rate of conversion of amino acids into protein. The final level of protein is however very dependent on environmental conditions during grain growth. For example, Mather and

Giese (1984) showed that the endosperm nitrogen and protein content of cultured barley ears increased with increase in nitrogen supplied. Similarly, Bottacin, Smith, Miflin, Shewry and Bright (1985) have found that increasing the glutamine supplied to cultured barley caryopses stimulated hordein accumulation. Potassium has been found to promote wheat grain protein synthesis by increasing the rate of amino acid translocation into the grain and their conversion to proteins (Mengel, Secer and Koch, 1981). In intact plants, the grain nitrogen content as a percentage of dry weight was found to increase with temperature by some workers, although the actual quantity is lower at high temperatures (e.g. 30°C) (Kolderup, 1975; Sofield, Wardlaw, Evans and Zee, 1977). However, Bhullar and Jenner (1985) found that while percentage nitrogen increased when exposed to a brief period at 33°C, the actual amount remained the same. Kolderup (1975) found that percentage nitrogen was also increased by longer photoperiod.

Protein deposits have been identified in wheat endosperm tissue by several groups of workers (e.g. Graham, Jennings, Morton, Palk and Raison, 1962; Buttrose, 1963; Graham, Morton and Raison, 1963; Jennings, Morton and Palk, 1963). They are surrounded by a single trilaminar membrane and are observed in the endosperm from about 7-10 days after anthesis onwards (Campbell, Lee and O'Brien, 1981; Bechtel, Gaines and Pomeranz, 1982b). The membrane may contain one or more granules of protein (Bechtel, Gaines and Pomeranz, 1982a) which vary in size between 5-100μm diameter (Pernollet and Camilleri, 1983).

The precise origin of the protein deposits is still unclear. The presence of polypeptide chains common to the endoplasmic reticulum and the protein deposits supports the theory that the storage proteins are first discharged into the endoplasmic reticulum prior to translocation to other parts of the cell (Pernollet and Camilleri,

1983). The ribosomes and polyribosomes of the rough endoplasmic reticulum have been proposed as the site of storage protein synthesis in wheat and barley endosperms (Graham *et al.*, 1962; Cameron-Mills and Ingversen, 1978; Donovan, Lee and Longhurst, 1982), with the proteins being formed inside the endoplasmic reticulum (Cameron-Mills and von Wettstein, 1980). Barlow, Lee and Vesk (1974) raised the possibility that free ribosomes were the site of synthesis early in development while the endoplasmic reticulum took over in the later stages. The ratio of bound to free polyribosomes has been shown to increase from 1 to 3 between 8 and 32 days after anthesis (Donovan *et al.*, 1982). The Golgi apparatus has also been implicated in the formation of protein deposits, possibly as a site of packaging (Bechtel and Gaines, 1982). Small vesicles were found by Buttrose (1963) which were associated with both the Golgi apparatus and the protein deposits, and this is supported by more recent work (Bechtel *et al.*, 1982b; Parker and Hawes, 1982).

Pernollet and Camilleri (1983) have put forward a model formation of protein deposits in which storage proteins are synthesised by polyribosomes into the lumen of the endoplasmic reticulum. They are transported via the Golgi apparatus into small vesicles which quickly associate into 4-10μm deposits and then move slowly into vacuolar-like structures in which they associate into huge clusters up to 75-100μm in diameter. Disruption of the membrane results in the formation of the protein matrix at later stages of grain maturation.

1.7 Plant Growth Regulators.

In higher plants, growth is a sequence of closely-controlled events thought to be regulated by plant growth regulators. Knowledge of the functioning of endogenous plant growth regulators in the control of grain development and termination of growth is very limited, partly due to the fact that concentrations are generally very low. It is not clear, for instance, whether responses are dependent on the concentration of a particular growth regulator, or just on its presence or absence, and it has been suggested that specific receptor sites might be required before it has any effect (Trewavas, 1981). Auxins, gibberellins, cytokinins and abscisic acid have all been identified in developing cereal grains, but most studies have not distinguished between the different tissues of the grain. The role of plant growth regulators in cereal grain development has been recently reviewed by Duffus (1985).

1.7.1 Cytokinins.

Cytokinins are thought to be synthesised in the roots and transported upwards in the xylem stream. It has been observed that the maximum level of cytokinin in the stem precedes that in the grains by a few days (Wheeler, 1972). The peak of cytokinin in the grains occurs immediately post anthesis dropping away to low levels by two to three weeks after anthesis. It has been suggested on the basis of these results that cytokinins are required for cell division as this correlates with the period of endosperm cell division (Wheeler, 1972). However. there is no direct evidence that this is so. Various studies have found that larger grains contain higher levels of

cytokinin (Michael and Seiler-Kelbitsch, 1972; Dua and Sehgal, 1981). One theory is that cytokinins enhance grain size by increasing cell numbers (Michael and Beringer, 1980) and, as was seen previously (Section 1.3.2), this is correlated with higher final grain dry weight. Interaction of cytokinins with auxin to promote cell division and differentiation has been reported (Duffus, 1985). These results suggest that cytokinins are associated with the initial stages of grain development. A role in the termination of dry matter deposition and maturation appears unlikely.

1.7.2 Auxins.

Auxin content shows a slight rise and fall around the period of anthesis, but the main peak is at about four to five weeks after anthesis which is a few days before the grain achieves its maximum fresh weight (Wheeler, 1972). All auxin activity disappears by maturity. Studies with rye show that the site of auxin synthesis is probably within the ear (Hatcher, 1943). The role of auxins in grain development remains obscure, but Radley (1979) has suggested that they might play a role in the inhibition of precocious germination.

1.7.3 Gibberellins.

Gibberellin levels in the grain peak about midway through the grain filling period (Wheeler, 1972) and are fairly equally divided between the endosperm and surrounding layers (Radley, 1976b). The increase in gibberellin levels has been associated with the cell expansion phase of grain development (Radley, 1976b). A possible role

for gibberellins in grain development is the regulation of assimilate accumulation (Wheeler, 1972; Mounla and Michael, 1973).

It has been shown that wheat ears are capable of synthesising gibberellins when detached from the plant (Radley, 1976a) although the precise location of synthesis is not known. During germination, synthesis is in the embryo but it is not clear how early the appropriate mechanism is functional. The green layer of the pericarp is another possible site of synthesis as there is evidence to suggest that chloroplasts are the site of gibberellin synthesis in some tissues (Stoddart, 1969). Radley (1976b) has proposed that gibberellins are required to keep the growth of the various tissues of the caryopsis in step, and that they might therefore be synthesised in different tissues at different stages of development. However, no mechanism whereby this could be achieved was proposed.

1.7.4 Abscisic Acid.

There has been considerable interest in the possible role of abscisic acid (ABA) in the regulation of grain development. Free ABA levels of several cereal grains increase rapidly in the later stages of grain filling, achieving a maximum at the point where grain filling ceases and there is onset of maturation (Goldbach and Michael, 1976; Radley, 1976b; King, 1976; King, Salminen, Hill and Higgins, 1979). Most of this is associated with the endosperm and pericarp (Radley, 1976b), with absolute levels dependent on environmental factors such as temperature and water stress (Radley, 1976b; Goldbach and Goldbach, 1977). A brief rise in the amount of bound ABA in barley grains just after the onset of water loss has also been observed (Slominski, Rejowski, and Nowak, 1979).

ABA is usually assigned a growth inhibitory role. In grain development and maturation it has been implicated in a variety of events, some inhibitory, others with a stimulatory role. Regulation of maturation could depend on both types of function (Karssen, 1982). The correlation between maximum ABA and the decline in water content and growth rate has suggested a regulatory function in the termination of growth (King, 1976). King found that application of ABA to ears close to maturation hastened the onset of water loss. Another possible role of ABA is in the inhibition of germination. Dunwell (1981) found that germination of isolated barley embryos, which was promoted by gibberellic acid, was totally prevented by ABA. Conversely, Black (1980/81) found no clear relationship between ABA levels and germinability in wheat grains although, as he indicated, the embryonic levels could be more significant. It is likely that germination is in fact controlled by interaction of ABA and gibberellins (Duffus, 1985). It has also been found that if ABA is injected into grains in the early stages of grain filling , when endogenous levels are low, there is an increase in the flow of assimilates into the grain (Dewdney and McWha, 1978; Tietz and Dingkuhn, 1981; Tietz, Ludewig, Dingkuhn and Dorffling, 1981). A short-term increase in sucrose uptake was also observed when ABA was applied to the glumes (Radley, 1981). However, if ABA was injected when the endogenous levels were high, assimilate uptake was strongly inhibited (Tietz and Dingkuhn, 1981; Tietz, Ludewig, Dingkuhn and Dorffling, 1981). These authors proposed that ABA is involved in the regulation of assimilate supply, possibly by the promotion of sieve tube unloading. It may be that interaction between ABA and sucrose levels is an important factor in the termination of starch deposition.

The origin of ABA is obscure. Experiments using foliar applied ^{14}C-ABA have shown that some is recovered from the ear. Goldbach and Goldbach (1977) took this as an indication that ABA is translocated to the grains from the leaves, although they did not rule out the possibility of additional synthesis in the ear, particularly in the glumes and awns. Dewdney and McWha (1978) carried out similar experiments but concluded that translocation was not the major source as the levels recovered in the grain were very low. This agrees with the results of Milborrow and Robinson (1973) who demonstrated sufficient mevalonate metabolism in the grain to account for all the ABA found. Synthesis of ABA within the ear is supported by changes similar to those occurring in intact ears being observed in detached ears cultured on ABA-free medium (King, 1979).

Assigning a specific function to a particular plant growth regulator has proved to be very difficult, which is partly due to the low levels in the tissues previously mentioned. Also, much of the evidence is indirect, based on the response of tissues to which the growth regulator has been applied. From the work discussed above, it appears that abscisic acid is the most likely candidate for a role in the termination of dry matter deposition, possibly interacting with gibberellins for the control of dormancy and germination.

1.8 Water Relations.

Water content of the grain is another factor which it is thought may have a role in the regulation of grain development and termination of growth. The water content of a developing cereal grain follows a typical pattern. It increases in the first two to three weeks after

anthesis during the cell division phase. There is then the period of grain-filling when water content changes little, followed by the final maturation phase when grain-filling has ceased and the grain rapidly loses water (Meredith and Jenkins, 1975; Sofield, Wardlaw, Evans and Zee, 1977; Barlow, Lee, Munns and Smart, 1980).

Entry of water into the grain from the rachilla involves circumvention of the xylem discontinuity at the base of the grain. This has been recently studied by Jenner (1985a; b; c) using tritiated water (THO). Little or no net transfer of water through the rachilla during assimilate uptake indicates that efflux balances influx, lending weight to the theory that active removal of water is occurring (Jenner, 1985a). Methods whereby the import of assimilate was slowed were found to increase the inflow of THO, implying that mass flow of assimilate and water is not the only or even the primary method of entry into the grain (Jenner, 1985b). Diffusion was found to be sufficient to account for the observed influx of THO into normally functioning grains (Jenner, 1985c). However, the rate was far slower than was predicted from the rate of diffusion of THO in water suggesting that there are considerable resistances to entry. A high degree of resistance to diffusion of water could explain the apparent hydraulic isolation of the grain from the rest of the plant observed by other groups (Barlow *et al.*, 1980; Brooks, Jenner and Aspinall, 1982). These groups found that water stresses which resulted in decrease of water potential in other parts of the plant had little effect on the grain water potential. Recycling of water within the caryopsis, as was proposed by Jenner (1982b) and Cochrane (1983), could also help to maintain its hydraulic independence.

The pathway of water entry into the endosperm from the crease vascular bundle is thought to be symplastic with solute assimilates.

Various mechanisms for the loss of water from the grain have been proposed. Lee and Atkey (1984) measured transpiration losses and concluded that these were sufficient to maintain a constant water level during grain filling using a calculation based on a hypothetical solute concentration entering the grain. Cochrane (1983) has proposed that water loss is via an apoplastic pathway returning through the chalazal region. She suggested that the xylem parenchyma cells might be involved in the active removal of water as they appear to remain metabolically active until well into the maturation period. They contain the raw materials necessary to raise the sucrose concentration in their cell walls so that the concentration is above that of the nucellar projection. Thus, water could leave down a concentration gradient. As well as this active removal, water could also be lost by passive diffusion through the pericarp and via the stomata at the apical end.

The final loss of water during the maturation phase is probably a result of blockage of the symplastic pathway in the chalazal region, while the apoplastic pathway is still able to let water pass. Sofield, Wardlaw *et al.* (1977) observed that entry of calcium, which they used as a marker for water entry, ceased when dry matter accumulation stopped. It is not clear whether the cessation of dry matter deposition is a result of the onset of water loss. The cause of the loss of water is not known, although ABA has been implicated here (Section 1.7.4).

1.9 Termination of Grain Growth.

As was noted in Section 1.1, termination of grain growth has been defined as the point at which dry matter deposition ceases. Little or no specific work on the termination of grain growth has been published, although many of the events occurring around the termination period have been described. The aim of this work is to compare processes during grain development and to try to identify some of those which are important in the termination of dry matter accumulation. The order in which they occur would be critical in establishing a pathway of cause and effect, and the key events might then be potential targets for manipulation of grain development. This is one way in which the final yield and quality could be improved.

Wheat was selected for this study due to its economic importance in the world food industry, as outlined in Section 1.1. The absence of a husk also facilitates the dissection of the grain. Spring wheat cultivars were used as the lack of a vernalization requirement makes them more convenient to grow in a glasshouse than winter wheat cultivars.

2. GRAIN DEVELOPMENT.

2.1 Introduction.

A major problem when comparing results on grain development with those from other groups of workers is that the time-scale of development varies. For example, the time taken from anthesis to maturity is about 37 days in the state of Washington, U.S.A. (Lingle and Chevalier, 1985), whereas in Scotland we have found it is about 60 days. In addition, this varies between seasons. It is well documented that environmental factors such as temperature affect the duration of development (e.g. Sofield, Evans, Cook and Wardlaw, 1977; Chowdhury and Wardlaw, 1978). Ideally, experiments should be carried out in controlled environment chambers. However, this is not always possible and there would still be variations between the cabinets used, albeit to a lesser extent. It is also possible that constant conditions may present problems as they are not representative of the true environment in the field.

To overcome this and to permit comparison between different experiments, some sort of developmental "ageing" scale is required to which all plant material can be referred. The requirements would be that it is quick, reproducible, preferably non-destructive and does not require large numbers of grains.

In this Department, a carefully described and recorded sequence of changes in morphological characteristics has been selected as a convenient way to "age" grains. This entails measurement of the size of parts of the caryopsis and a description of tissue appearance. A scale based on a field trial in 1983 of spring wheat cv. Sicco is

shown in Tables 2.1 and 2.2 (Riffkin, personal communication). This has the advantage that all glasshouse material can be "aged" with reference to field grown material. A similar developmental scale has been published by Rogers and Quatrano (1983), but this is considerably less detailed and was based on plants grown in a glasshouse and transferred to a growth chamber at anthesis. Field-grown material was not used.

The purpose of the work described in this chapter is to relate key yield-controlling factors to a known and reproducible stage of caryopsis development. In order to understand the events leading to the termination of dry matter deposition, it is clearly essential to record the gross changes accompanying development from anthesis to harvest-ripeness. Thus, the following measurements were made in order to establish the overall pattern of events accompanying grain development. Dry weights, which are a fundamental indicator of yield, were recorded together with fresh weights. From these determinations, the water content of caryopses and endosperms over the developmental period could be estimated. Storage products are of importance in yield determination, and so changes in the levels of starch and total nitrogen, which relates to protein content, were monitored in the endosperm. Sucrose, as the precursor to starch was also of interest, and was measured to determine whether levels of this could be affecting starch deposition at any stage of development. The inter-relationships of these measurements are important as a preliminary study of some of the events at termination of dry matter deposition. Data for caryopsis fresh and dry weights and endosperm starch content were also recorded for field-grown material to check that results from glasshouse-grown plants were relevant to the field situation.

TABLE 2.1.

Developmental Scale from Field-Grown Wheat cv. Sicco 1983.

Tissue Sizes and Brief Description.

(H.L. Riffkin, personal communication).

Age-days after anthesis	Description of Pericarp	Size of Caryopsis (mm)	Size of Embryo (mm)	Size of Endosperm (mm)	Description of Endosperm
1	Very pale green Crease light green	1.0 x 2.0			
2	Pale green	1.2 x 2.0			Transparent, liquid
5	Pale green	3.0 x 2.5			Transparent, liquid
9	Pale green Crease green	4.0 x 2.5			Transparent, liquid
12	Pale green	5.3 x 3.5		4.5 x 3.3	Opaque, fluid very fragile
16	Pale green	6.0 x 3.5	Pin tip	5.5 x 3.5	Cream, soft moist contents
19	Light-medium green	6.0 x 4.0	1.0 x 1.0	5.8 x 4.0	Cream, soft moist contents
23	Medium green	6.3 x 4.0	1.8 x 1.2	6.2 x 4.0	White, moist becoming sticky
26	Green	6.5 x 5.0	2.5 x 2.0	6.0 x 4.5	Milk-white, moist-firming
30	Green	6.5 x 5.0	2.5 x 2.0	6.0 x 4.8	Milk-white, moist dough
33	Green-beginning to yellow	6.5 x 5.0	3.0 x 2.0	6.5 x 4.8	Milk-white, soft-doughy
37	Yellowing-green	6.5 x 5.0	3.0 x 2.0	6.5 x 5.0	White, sticky dough
40	Yellow, crease still green	6.5 x 5.0	3.0 x 2.0	6.5 x 5.0	Cream, drying dough
44	Yellow-brown pale green crease	6.3 x 4.5	3.0 x 2.0	6.0 x 4.5	Cream, firm, imprint partially remains
47	Mid-brown, slight wrinkling	6.3 x 4.0	3.0 x 2.0	6.0 x 4.0	Cream, dry, rubbery. Imprint remains
†51	Red-brown	6.0 x 3.5	3.0 x 2.0		Cream, hard, floury becoming brittle

†Caryopsis colour function of testa (pericarp is transparent).

TABLE 2.2.

Developmental Scale from Field-Grown Wheat cv. Sicco 1983.

Detailed Description of Tissues.

(H.L. Riffkin, personal communication).

Age-days after anthesis	Shape	Appearance of pericarp transparent (TL) and green (GL) layers	Fusion GL / Testa	Description of Embryo	Description of Endosperm
1		TL/GL not clearly differentiated	No		(Ovule) clear, liquid
2		TL/GL just separable, TL light, fluffy	No		Clear, liquid
5		TL/GL separate with difficulty, TL fleshy, moist	No		Liquid, no shape
9		TL/GL separate, TL fleshy, moist, GL green	No		Liquid, fragile
12		TL/GL separate cleanly, TL medium moist	No		Shallow cheeks, can be squeezed out
16		TL/GL separate cleanly TL medium moist	No	Pin tip	Cheeks start to fill, dorsal area thin
19		TL/GL separate easily, TL medium moist	No	Pale, fragile	Cheeks filling, dorsal thickening
23		TL/GL separate easily TL medium-thin, moist	No	Moist, soft	Cheeks filled, dorsal area thick
26		TL/GL separate easily TL becoming thin	Slight	Moist firming	Cheeks fattening, dorsal area thick
30	"	TL/GL separate easily, TL thin, moist	Partial	Light yellow firm	Cheeks and dorsal area fattened
33	"	TL/GL sticky separation, TL thin, drying	Partial	Yellow, firm	Cheeks and dorsal area fattened
37	"	TL/GL sticky separation TL thin, drying	Yes	Yellow, firm, crisp	Cheeks and dorsal area fattened
40		TL/GL sticky separation TL thin, drying	Yes	Yellow, firm, crisp	Cheeks and dorsal area fattened
44		TL/GL difficult to separate, TL flakey	Yes	Yellow, firm, crisp	Firm, imprint partially remains
47	Slight wrinkling	TL/GL difficult to separate, TL flakey	Yes	Yellow, firm crisp	Dry, rubbery, imprint remains
51	Shrunken appearance	TL/GL difficult to separate, TL flakey	Yes	Firm, oily	Hard, floury, becoming brittle

2.2 Materials and Methods.

2.2.1 Plant Material.

Wheat plants, *Triticum aestivum* cv. Sicco, were grown by the Glasshouse Unit, Edinburgh School of Agriculture. Six plants per pot were grown in Levingtons compost in 20cm diameter pots. Natural light was supplemented with 400W mercury vapour lamps to give an 18h photoperiod, and the day/night temperature was approximately 20/12°C. Plants were watered daily, and fed with a liquid feed containing potassium nitrate, ammonium phosphate and ammonium nitrate (55g, 20g and 45g per litre of stock, diluted 1: 600 for use) three times per week. Insect and fungal infections were minimised by spraying with Pirimor (I.C.I.) and Bayleton (Bayer) as required.

Caryopses were "aged" according to a developmental scale based on a 1983 field trial for Sicco (Riffkin, personal communication). The age on the scale was recorded as "days" after anthesis. The details of this scale, which is based on changes in morphological characteristics, are shown in Tables 2.1 and 2.2. For all work presented in this thesis, only caryopses from the a and b positions of the five central spikelets on each side of main ears or primary tillers were used.

Plants required for field experiments were grown in trial plots on the Bush Estate by the Crop Production Department, Edinburgh School of Agriculture.

2.2.2 Fresh and Dry Weights.

Samples of five caryopses were taken for fresh weight determinations, then oven dried to a constant weight at 80°C. Water content was calculated from these results.

Fresh and dry weights of endosperms were obtained similarly, using endosperms dissected from caryopses.

2.2.3 Sucrose Determination.

Endosperms were dissected from caryopses and retained on moist filter paper in a Petri dish until sufficient were prepared for an extraction. Sucrose was extracted by homogenising endosperms (8-12) in 1ml ice-cold 0.25M perchloric acid. The homogenate was diluted four-fold with ice-cold double-distilled water and centrifuged at 5000g for 10 min at 0-4°C. The supernatant was removed and retained on ice, and the pellet was re-extracted in 0.5ml 0.25M perchloric acid, diluted and centrifuged as before. The supernatants were combined, neutralised with 5M potassium hydroxide, recentrifuged and the final supernatant retained.

The method of assay was a modification of that of Bergmeyer and Bernt (1974). A sample aliquot of 200µl was mixed with 100µl β-fructosidase solution (β-fructosidase, Boehringer Co. Ltd., 5mg/ml in 0.32M citrate buffer pH4.6) and 100µl 0.32M citrate buffer pH4.6 and incubated for 15 min at 25°C. Glucose released by sucrose inversion was determined using the hexokinase / glucose-6-phosphate dehydrogenase method of Bergmeyer, Bernt, Schmidt and Stork (1974). A 0.2ml sample of the β-fructosidase incubation was added to 1ml of 0.2M triethanolamine-HCl buffer pH8.0, containing 1.5mM $MgCl_2$, 100µg/ml BSA (fraction V), 0.56mM ATP and 0.33mM NADP. The absorbance at 340nm was

recorded on a Pye Unicam SP8-100 UV/Vis spectrophotometer. A_{340} was read again after incubation for 15 min at 25°C with 5µl hexokinase / glucose-6-phosphate dehydrogenase (Boehringer Ltd.), and the results calculated using the molar extinction coefficient for NADP (ϵ=6.3m^2mol^{-1}). Glucose present in the sucrose preparation was determined by omitting the inversion step. Internal sucrose standards were used to check recovery. Known amounts of sucrose were added to some samples and the results were compared with replicates of the same samples to which sucrose had not been added.

2.2.4 Starch Determination.

Starch was extracted and assayed by a modification of the method of Ahluwalia and Ellis (1984). Starch was extracted by homogenising endosperms (3-5) in 0.05M perchloric acid and incubating at 96°C for 3 min. When cool, the sample was made up to 100ml with distilled water. 250µl Aliquots were digested with 200µl pronase (Boehringer Ltd., 2.5mg/ml in 0.2M Tris, 0.1M NaCl buffer pH7.2) with an extra 200µl Tris-NaCl buffer at 30°C for 30 min and then boiled for 1 min. A 100µl aliquot from this incubation was added to 0.9ml 0.2M sodium acetate buffer pH 4.8 and digested with 20µl amyloglucosidase (Boehringer Ltd., 14U/mg, 10mg/ml) at 55°C for 40 min. Glucose determination was according to Bergmeyer *et al.* (1974), as described in Section 2.2.3, but using a 0.3ml sample. Controls for glucose present in the preparation were determined by omitting the amyloglucosidase digestion. Known amounts of commercial wheat starch were also extracted by this method and used to determine percentage recovery.

2.2.5 Nitrogen Determination.

This was by the semi-micro-Kjeldahl method (Gough, 1981). Endosperms (5-6) were digested in 5ml concentrated sulphuric acid with 1g Kjeldahl catalyst (3:1 potassium sulphate : copper sulphate) by boiling on a Kjeldahl digestion unit until a clear blue colour was observed, and then for a further hour. After cooling, the sample was diluted to 25ml or 50ml with distilled water. A 5ml aliquot was distilled with 5ml 5M sodium hydroxide in a Hoskins steam distillation unit, and the ammonia collected in 10ml 0.16M boric acid and titrated against M/140 hydrochloric acid using Tashiro's indicator. Blank controls in which sucrose was used as the sample were performed, and recovery was checked using a standard solution of 10mM ammonium sulphate.

2.3 Results.

For all results presented graphically in this thesis, bars represent the limits of standard error. They are omitted where they are too small to present clearly. All results presented are the mean of at least three replicate samples. Where horizontal axes represent "days" after anthesis, this is abbreviated to daa.

A photograph of the pot system used for growing plants in the glasshouse is shown in Fig. 2.1. Field trial plots on the Bush Estate are shown in Fig. 2.2. Representative samples of ears and caryopses of cv. Sicco at different stages of development are shown in Figs. 2.3 - 2.8. The stage of development in "days" after anthesis was determined using the developmental scale.

Fig. 2.1. Pot of spring wheat plants cv. Sicco as grown in the glasshouse.

Fig. 2.2. Trial plots of spring wheat cv. Sicco growing on the Bush Estate in 1984.

Fig. 2.3. Ear of wheat cv. Sicco at 25 "days" after anthesis.

Fig. 2.4. Ear of wheat cv. Sicco at 55 "days" after anthesis.

Fig. 2.5. Caryopsis of wheat cv. Sicco at 15 "days" after anthesis.

Fig. 2.6. Caryopsis of wheat cv. Sicco at 25 "days" after anthesis.

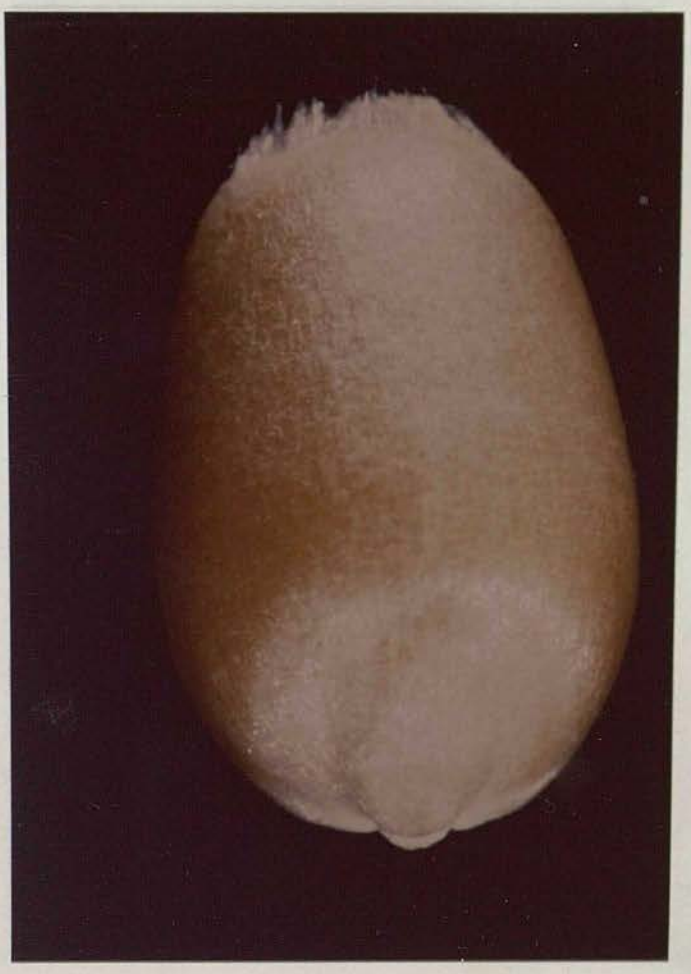


Fig. 2.7. Caryopsis of wheat cv. Sicco at 40 "days" after anthesis.

Fig. 2.8. Caryopsis of wheat cv. Sicco at 55 "days" after anthesis.

Fresh weight of both caryopses (Fig. 2.9a) and endosperms (Fig. 2.9b) shows a steady increase until about 35 "days" after anthesis and reaches a maximum at 40 "days", after which it declines until maturity. Dry weight of the caryopsis shows an initial lag until about 15 "days" after which it increases steadily to a maximum at 40 "days". Endosperm dry weight also shows a steady increase over the same period. Results for endosperms outside the range 15-45 "days" are not given as at ages younger than 15 "days" much of the endosperm contents are fluid, and at ages older than 45 "days" it is not possible to separate precisely the endosperm from the outer layers of the caryopsis which adhere to it. Water content of caryopses and endosperms (Fig. 2.9c) is highest between 30 and 35 "days" after anthesis, after which a steady decline is seen.

Sucrose levels (Fig. 2.10) were lowest at 15 "days" after which they increased and then fluctuated between 0.55 and 0.8μmoles per endosperm throughout the period of measurement. There was no significant change between 30 and 45 "days". Starch (Fig. 2.11) and nitrogen (Fig. 2.12) content show similar patterns of increase throughout development until 40 "days" after anthesis, after which the level is maintained.

Maximum fresh weight per caryopsis from field-grown material was achieved at 35 "days" and maintained until 44 "days", after which it declined (Fig. 2.13a). Dry weight reached a plateau at 40 "days". Water content per caryopsis (Fig. 2.13b) was fairly constant between 22 and 44 "days" before declining. Starch content per endosperm (Fig. 2.14) reached a plateau at 40 "days" after anthesis.

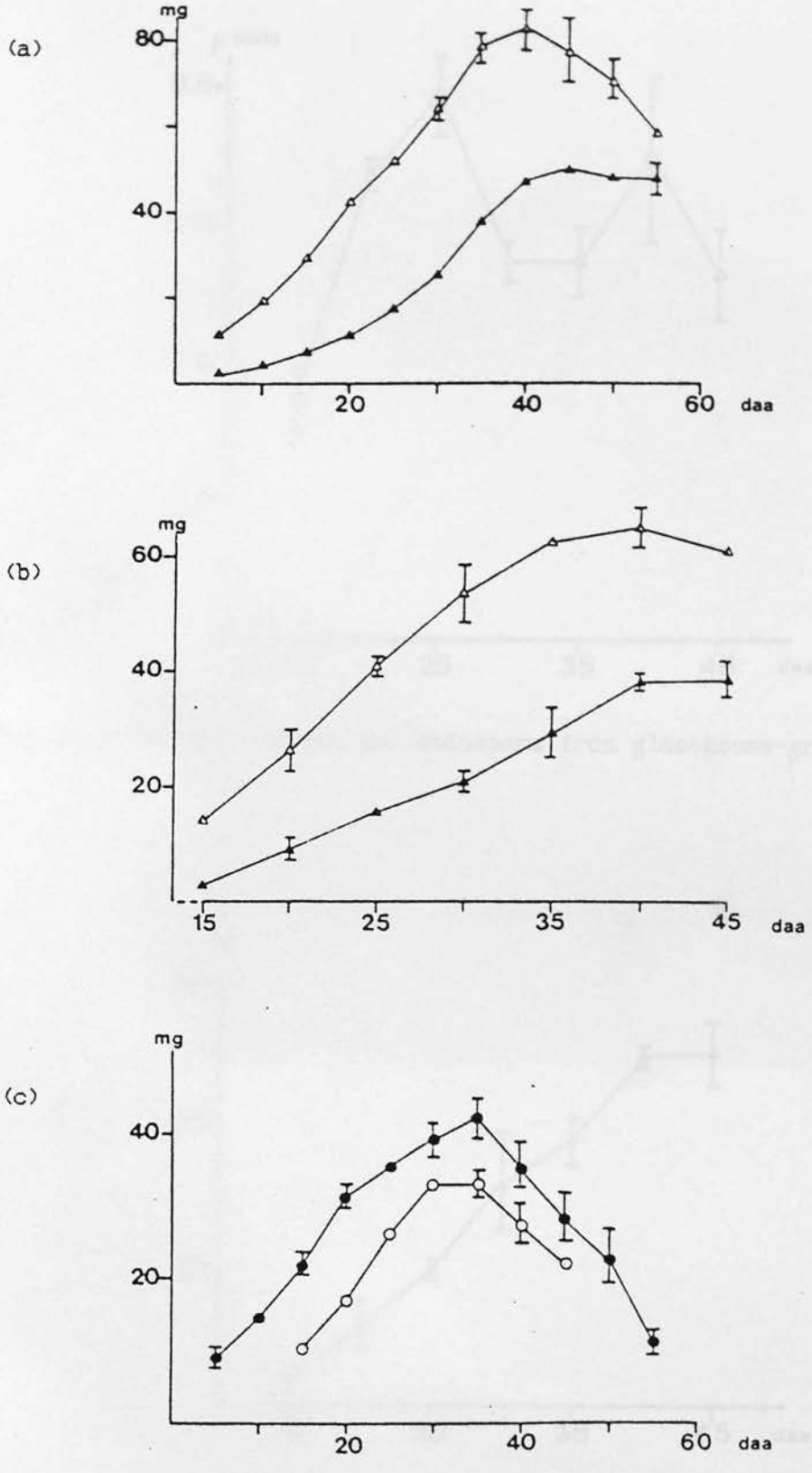


Fig. 2.9. Fresh weight (△) and dry weight (▲) (a) per caryopsis and (b) per endosperm; and (c) water content per caryopsis (●) and per endosperm (○) from glasshouse-grown plants.

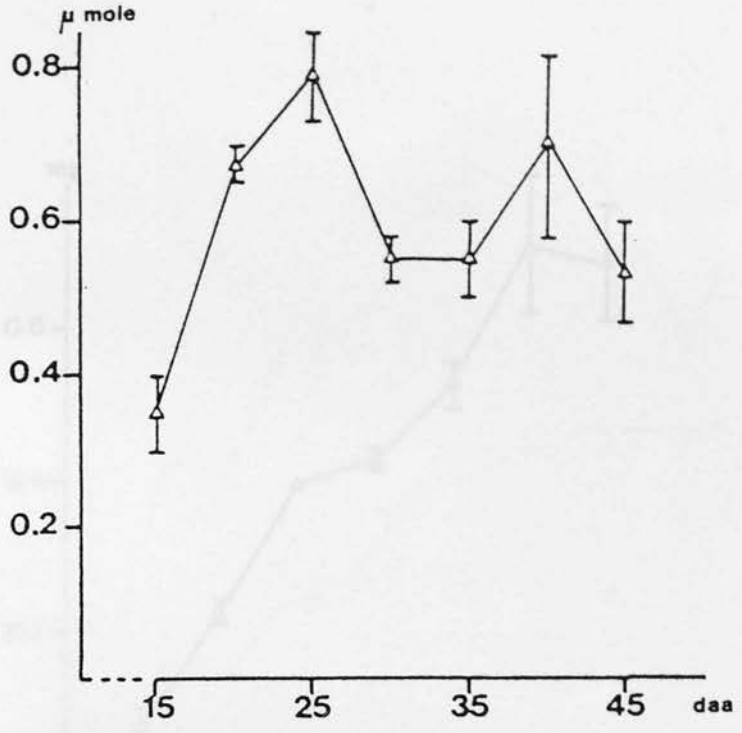


Fig. 2.10. Sucrose content per endosperm from glasshouse-grown plants.

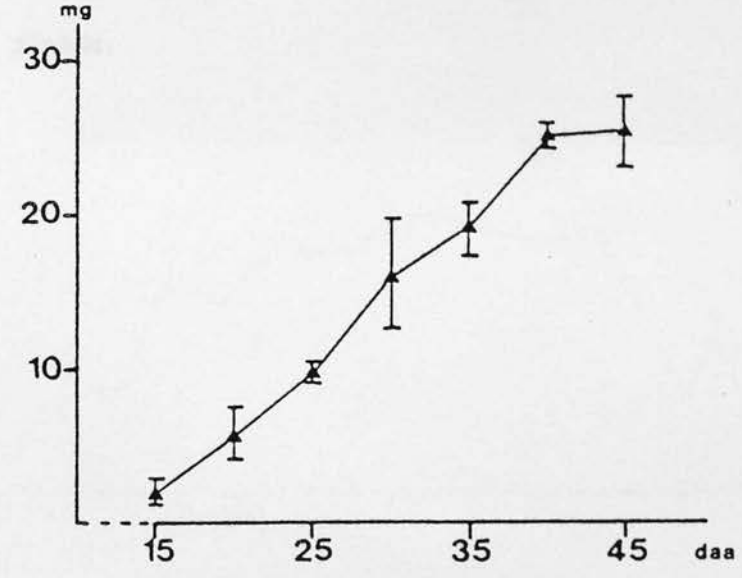


Fig. 2.11. Starch content per endosperm from glasshouse-grown plants.

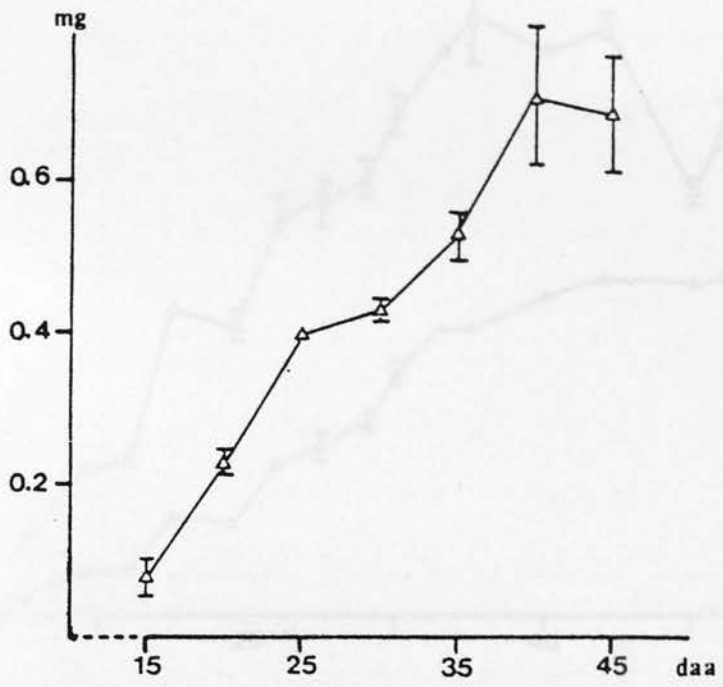


Fig. 2.12. Nitrogen content per endosperm from glasshouse-grown plants.

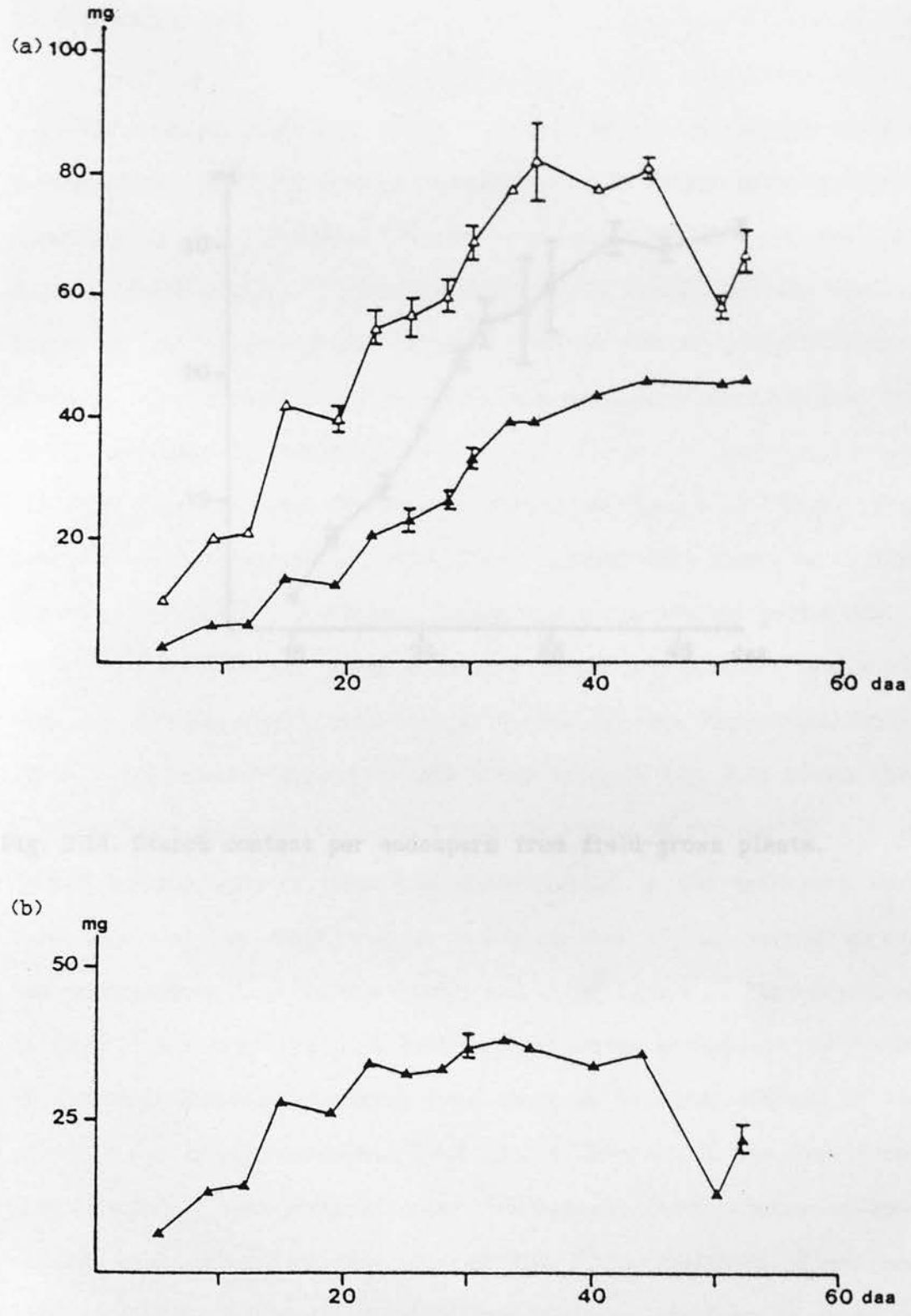


Fig. 2.13. (a) Fresh weight (△) and dry weight (▲) and (b) water content per caryopsis from field-grown plants.

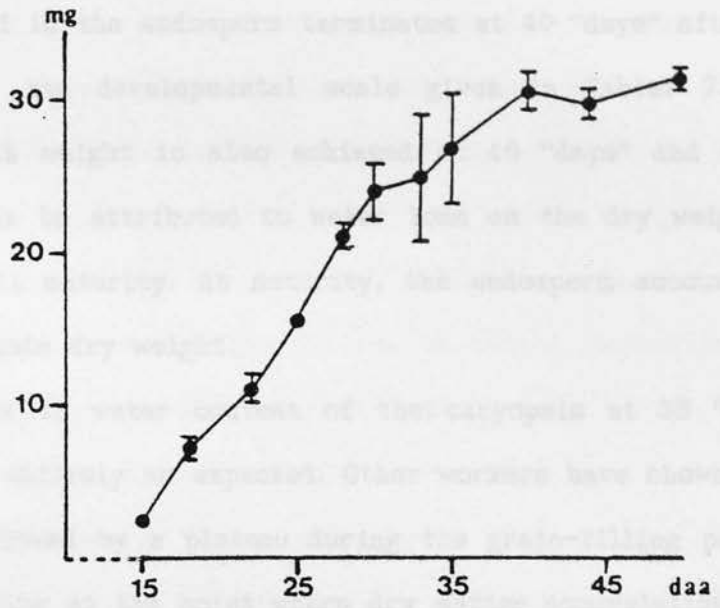


Fig. 2.14. Starch content per endosperm from field-grown plants.

2.4 Discussion.

The results presented show that dry matter deposition in the caryopsis and in the endosperm terminates at 40 "days" after anthesis according to the developmental scale given in Tables 2.1 and 2.2. Maximum fresh weight is also achieved at 40 "days" and the decline thereafter can be attributed to water loss as the dry weight remains constant until maturity. At maturity, the endosperm accounts for 76% of the caryopsis dry weight.

The peak in water content of the caryopsis at 35 "days" (Fig. 2.9c) is not entirely as expected. Other workers have shown an initial increase, followed by a plateau during the grain-filling period and a decline starting at the point where dry matter accumulation levels off (Sofield, Wardlaw, Evans and Zee, 1977; Barlow, Lee, Munns and Smart, 1980). However, overlapping of the error bars in Fig. 2.9c shows that in fact there may not be much significant change between 25 and 40 "days" and for part of this time water levels in the endosperm were also constant. The difference in water content of the caryopsis and the endosperm is that of the embryo and outer layers of the caryopsis. It is possible that the peak in caryopsis water content at 35 "days" is a true phenomenon resulting from increase in water content of the embryo and / or the caryopsis outer layers. However, it has been shown that drying of the pericarp outer transparent layer commences much earlier than drying of the rest of the grain (Mitchell, Black and Chapman, 1980), so it is unlikely that such an increase is found in this particular tissue.

Deposition of starch in the endosperm (Fig. 2.11) parallels the increase in dry weight of both the endosperm and caryopsis, which is in agreement with the results of Jennings and Morton (1963a) and

Cerning and Guilbot (1973). Starch contributes just under 70% of the final endosperm dry weight. Although the levels of sucrose in the endosperm (Fig. 2.10) show fluctuations during the period of starch deposition, there is no obvious decline to lower levels when starch accumulation ceases. This supports the view that availability of sucrose does not cause starch accumulation to cease. It also agrees with the work of Jenner and Rathjen (1977) with wheat and Cochrane (1985) with barley who showed that sucrose continued to reach the endosperm until after the termination of starch deposition. However, the results presented here do not distinguish between supply and turnover so it cannot be definitely stated that sucrose is continuing to enter the endosperm. One possibility is that sucrose levels remain constant because none is entering the endosperm and none is being used but this is very unlikely since starch synthesis is continuing.

Nitrogen accumulation (Fig. 2.12) also parallels the increase in dry weight, reaching a maximum at 40 days. The parallel increase agrees with the work of Feller (1978) and Gleadow, Dalling and Halloran (1982), but the results of Barlow, Lee and Vesk (1974) show total nitrogen continuing to increase after dry weight increase has levelled off. In their work, it is not shown which component of grain weight is decreasing at the same rate as nitrogen is increasing to allow for no alteration in dry weight. On the other hand, although Gleadow *et al.* (1982) show that nitrogen accumulation parallels dry weight increase, they did not extend the period of measurement after termination so it is unclear when nitrogen increase terminates. It is possible that the precise point at which nitrogen accumulation stops is partly dependent on cultivar. In the six wheat lines studied by Gleadow *et al.* (1982), the relationship of nitrogen content to dry weight increase varied, with the linear phase of nitrogen increase

continuing longer than the linear phase of dry weight increase in some cultivars but not in others.

Measurement of total nitrogen includes contributions from nucleic acids, storage proteins, soluble proteins and amino acids. Jenner (1979) estimated the protein content of whole caryopses by multiplying the nitrogen content less amino nitrogen by a factor of 5.7. This was presumably derived from protein and nitrogen measurements in replicate samples although it was not explained. If it is employed here, a maximum of 3.99mg of protein per endosperm at 40 "days" can be calculated, which gives a protein content of 10.5%. Given that the origin of the conversion factor is obscure and that the composition of the nitrogen source differs, this is not too dissimilar from the average figure of 12-13% protein for the caryopsis given by Duffus and Slaughter (1980).

Results from field-grown material were similar to those from the glasshouse. Fresh and dry weights of field-grown caryopses were slightly lower than those from the glasshouse, but starch content was slightly higher. Maximum dry weight was reached at 44 "days", a little later than for the glasshouse-grown material, although the increase after 40 "days" was slight. Starch levels in the endosperm did not increase after 40 "days". Fresh weight and water content did not start to decline until 44 "days" which is not consistent with the results from the glasshouse where water content started to fall slightly before dry weight increase stopped. This can be explained by the fact that inclement weather at this stage of the field trial led to surface water on the caryopses which tended to affect fresh weight determinations, and therefore calculated water content, even after blotting dry. More of a plateau in the water content was observed for the field-grown caryopses than had been for the glasshouse-grown

ones. The close similarity between the glasshouse and field results shows that results from glasshouse material are relevant to growth in the field and therefore applicable to the agricultural industry.

The results indicate that starch, nitrogen and dry matter accumulation terminate at the same point. It appears that this is slightly preceded by the onset of water loss, at least in the endosperm, rather than water loss commencing at the same time as termination as has been indicated previously by other workers. Therefore, it is possible that lowering of water levels beyond a critical point may cause the termination of dry matter accumulation.

3. CARYOPSIS CULTURE.

3.1 Introduction.

In recent years, a method for growing detached cereal ears in liquid culture, first used by Jenner (1968a), has been developed by Donovan and Lee (1977). Using this technique, the conditions of growth and the components of the growth medium can be manipulated to investigate their effects on grain growth and development.

The possibilities of caryopsis culture as a method of studying direct effects on caryopsis growth have been investigated. Gifford and Bremner (1981a) developed a method for wheat caryopsis culture which involved the partial submerging of grains in the culture medium following removal of the transparent layer of the pericarp to facilitate uptake. This method was used for culture periods of up to one week. Half grains were also cultured for periods of a few days. It is not clear how successful this method was compared to growth on the plant since dry weight determinations were not performed and no attempt was made to compare growth of the cultured caryopses with intact caryopses over the same period. Growth was only monitored by changes in the levels of carbon-14 in the ethanol-soluble and insoluble fractions of the caryopsis when cultured on ^{14}C-sucrose. The optimum sucrose concentration for growth was found to be 6-7%.

A similar method has been used by workers at Rothamsted Experimental Station (Bright, personal communication, later published as Bottacin, Smith, Miflin, Shewry and Bright, 1985) by which barley grains were cultured for 20 days. These workers did monitor dry weight increase in the cultured caryopses, as well as starch, sucrose

and protein content, but again there was no attempt made to compare growth with that of caryopses from an intact plant. Sucrose concentrations in the range 10-25% were used, but no optimum value was found. This is consistent with the small amount of culture work performed with barley caryopses in this Department in which no optimum sucrose concentration was found over the range 2-18% (Craig, 1983).

The main disadvantage of these methods is that they require the caryopsis to be covered in a film of the growth medium, and entry can be through any available opening. Thus, the environment for growth is entirely dissimilar to that thought to operate *in vivo*. In the method developed for this project, caryopses were not immersed in the growth medium, but were positioned vertically, with the intention that assimilate entered via the rachilla and the pathway of assimilate uptake followed that thought to operate in the intact ear. With this technique, it was hoped that any mechanisms controlling the uptake of assimilate *in vivo* would be present in the *in vitro* culture system.

A alternative method has been devised by Shimamoto and Nelson (1981) for culture of maize kernels. Groups of 4 kernels are supported on blocks of cob stem tissue which is in the growth medium. This block of tissue keeps the actual kernel out of the medium. Unfortunately, this is not a method which can be applied to wheat.

The intention of this work was that, if the technique were successful, caryopsis culture could be used as a tool whereby composition of the growth medium could be altered and the effect on caryopsis development investigated.

3.2 Materials and Methods.

3.2.1 Plant Material.

Wheat, *Triticum aestivum* cv. Sicco, was grown as described in Section 2.2.1. Caryopses were aged as described therein.

3.2.2 Growth Medium Composition and Sterilization.

The growth medium was prepared according to the method of Donovan and Lee (1977). This is essentially an adaptation of the plant tissue culture medium of Murashige and Skoog (1962) and Linsmaier and Skoog (1965). Stock solutions were made up as follows:

Major elements A - 4.9g $CaCl_2.2H_2O$ per 500ml.

Major elements B - 7.2mg $MgSO_4.7H_2O$ and 12.0g KH_2PO_4 per 500ml.

Minor elements - 6.2mg H_3BO_3, 10.6mg $ZnSO_4.7H_2O$, 0.25mg $NaMoO_4.2H_2O$, 0.025mg $CoCl_2.6H_2O$, 22.3mg $MnSO_4.4H_2O$, 0.83mg KI and 0.025mg $CuSO_4.5H_2O$ per litre.

Iron solution - 6.7117g ferric citrate and 7.45g Na_2EDTA per litre.

Vitamin solution - 0.004g thiamine-HCl and 1.0g myo-inositol per 100ml.

The complete medium comprised 50ml Major elements A, 50ml Major elements B, 10ml Minor elements, 5ml Iron solution and 10ml Vitamin solution per litre together with glutamine and sucrose at suitable

levels. Glutamine, at a concentration of 0.02M, was used as the sole nitrogen source (Donovan and Lee, 1978). Sucrose concentration was between 0.18 and 0.53M (6-18%). The complete medium was sterilized by passing through 0.2µm membrane filters.

3.2.3 Culture Method.

Caryopses were surface-sterilized under vacuum in 10% sodium hypochlorite solution and thoroughly rinsed in several changes of sterile distilled water. They were then supported in small indentations in pieces of sterile "Oasis" floral foam (80x55x8mm, approx., 20 caryopses per piece) contained in a sterile square petri dish, and soaked in sterile medium (25-30 ml per plate). All procedures were carried out in a laminar flow cabinet to reduce contamination.

The plates were incubated at ear height alongside intact plants in the glasshouse, under the conditions described in Section 2.2.1. The growth medium was replaced every 3 or 4 days and caryopses were surface-sterilized again. Sucrose concentration was maintained either at 6% (0.18M) or at 18% (0.53M), or increased from 6% to 10% to 14% to 18% at seven day intervals during the culture period.

3.2.4 Sampling Methods.

Caryopses were sampled at 5 day intervals for fresh and dry weights as described in Section 2.2.2. Caryopses from the same batch of plants used to provide the caryopses for culture were also sampled for comparison and the "age" was noted.

3.3 Results.

The system used for caryopsis culture is illustrated in Fig. 3.1. In the Figures and Tables, days refers to the number of days after the commencement of the culture experiment.

In the first experiment, caryopses at 20 "days" after anthesis were cultured on 6% sucrose medium for 15 days. The caryopses from the intact plants maintained a fairly steady rate of development during the period of the experiment as far as "age" on the developmental scale is concerned (Table 3.1). A similar pattern is seen from the fresh and dry weights (Fig. 3.2). In particular, the dry weights show a steady increase throughout the experiment, and this is paralleled by the cultured caryopses. However, the actual dry weight of the cultured caryopses at a given time is lower than that of the intact caryopses due to an initial lag when they were first put into culture.

In the second experiment, caryopses at 30 "days" after anthesis were cultured for 40 days on 6% sucrose, 18% sucrose or on medium in which the sucrose concentration was increased from 6% to 10% to 14% to 18% at 7 day intervals. The development of the intact caryopses in terms of "age" again shows a steady progression throughout the later stages of grain filling and the maturation period (Table 3.2). An increase in fresh weight and dry weight of the intact caryopses occurs for the first 10 days of the experiment (Fig. 3.3a). After this, the dry weight is maintained at a constant level while the fresh weight decreases.

The dry weights of caryopses cultured in all three sucrose regimes showed little change (Fig. 3.3b, c, d.). Slight increases in dry weight in the first 10 days are seen for the caryopses cultured on 6%

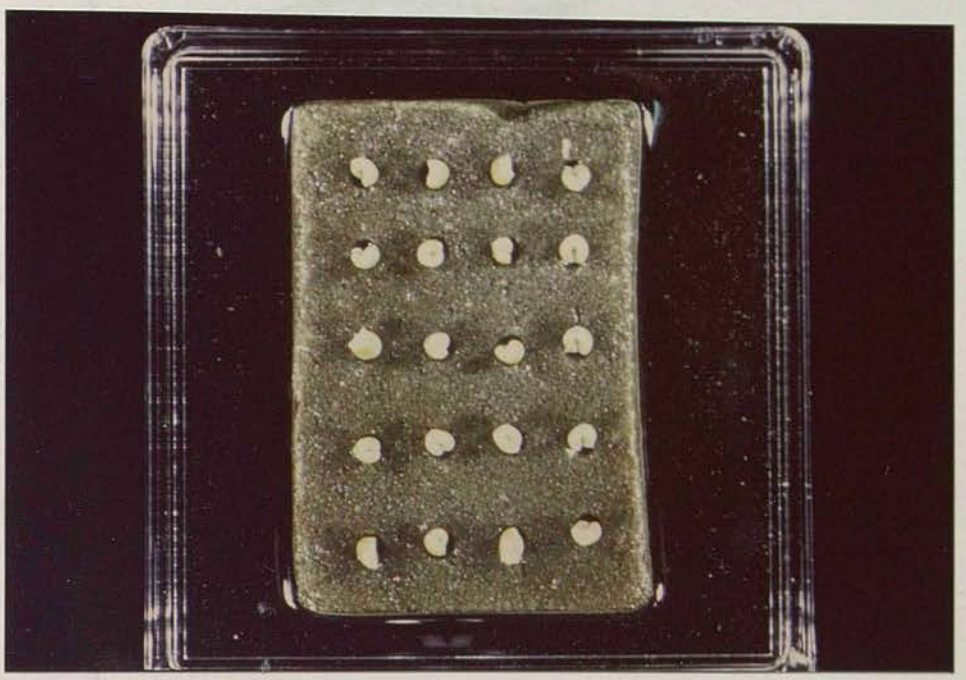


Fig. 3.1. Caryopses of cv. Sicco set up for caryopsis culture.

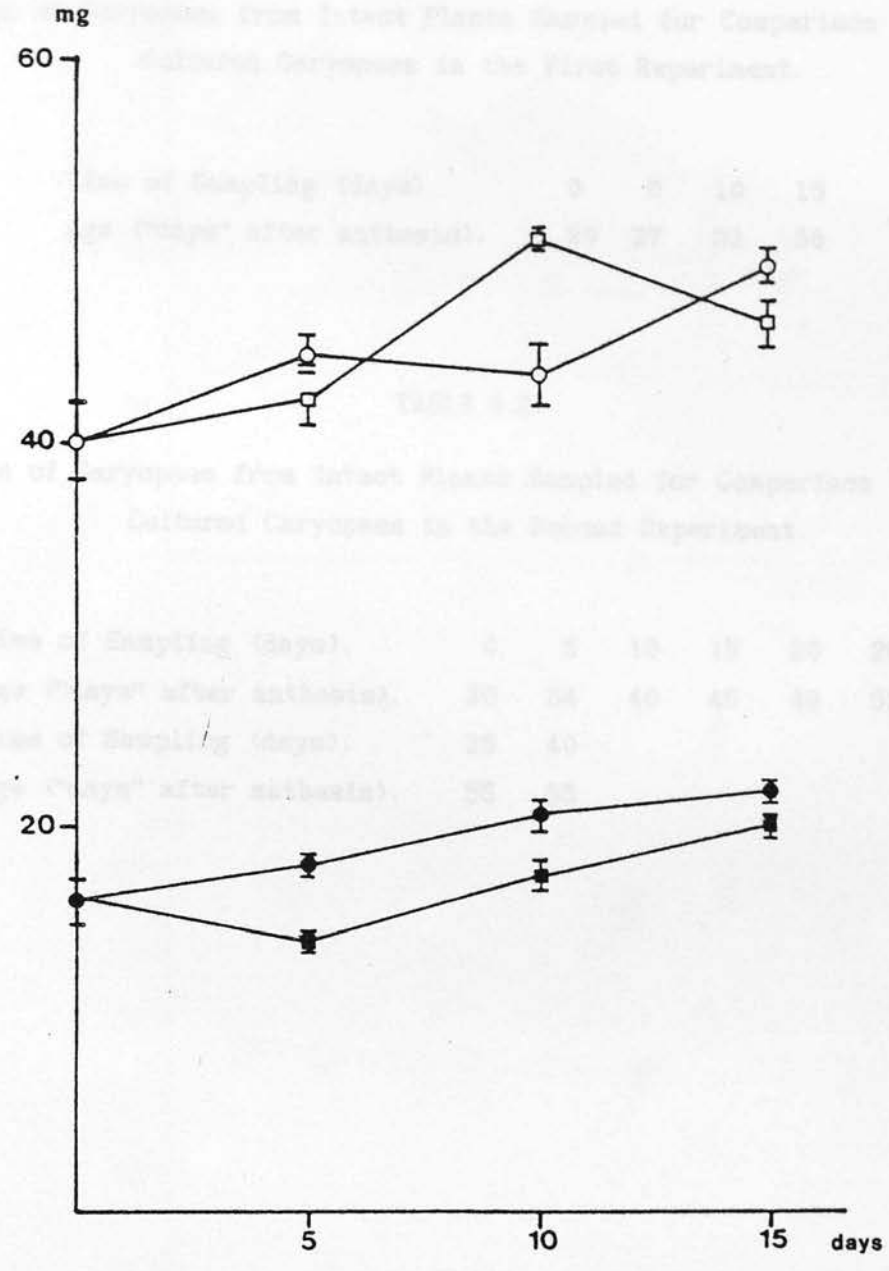


Fig. 3.2. Fresh weights (open symbols) and dry weights (closed symbols) of cultured caryopses (□,■) and caryopses from intact plants (○,●) in the first culture experiment.

TABLE 3.1.

Age of Caryopses from Intact Plants Sampled for Comparison with Cultured Caryopses in the First Experiment.

Time of Sampling (days)	0	5	10	15
Age ("days" after anthesis).	20	27	32	38

TABLE 3.2.

Age of Caryopses from Intact Plants Sampled for Comparison with Cultured Caryopses in the Second Experiment.

Time of Sampling (days).	0	5	10	15	20	25	30
Age ("days" after anthesis).	30	34	40	45	49	52	55
Time of Sampling (days).	35	40					
Age ("days" after anthesis).	55	55					

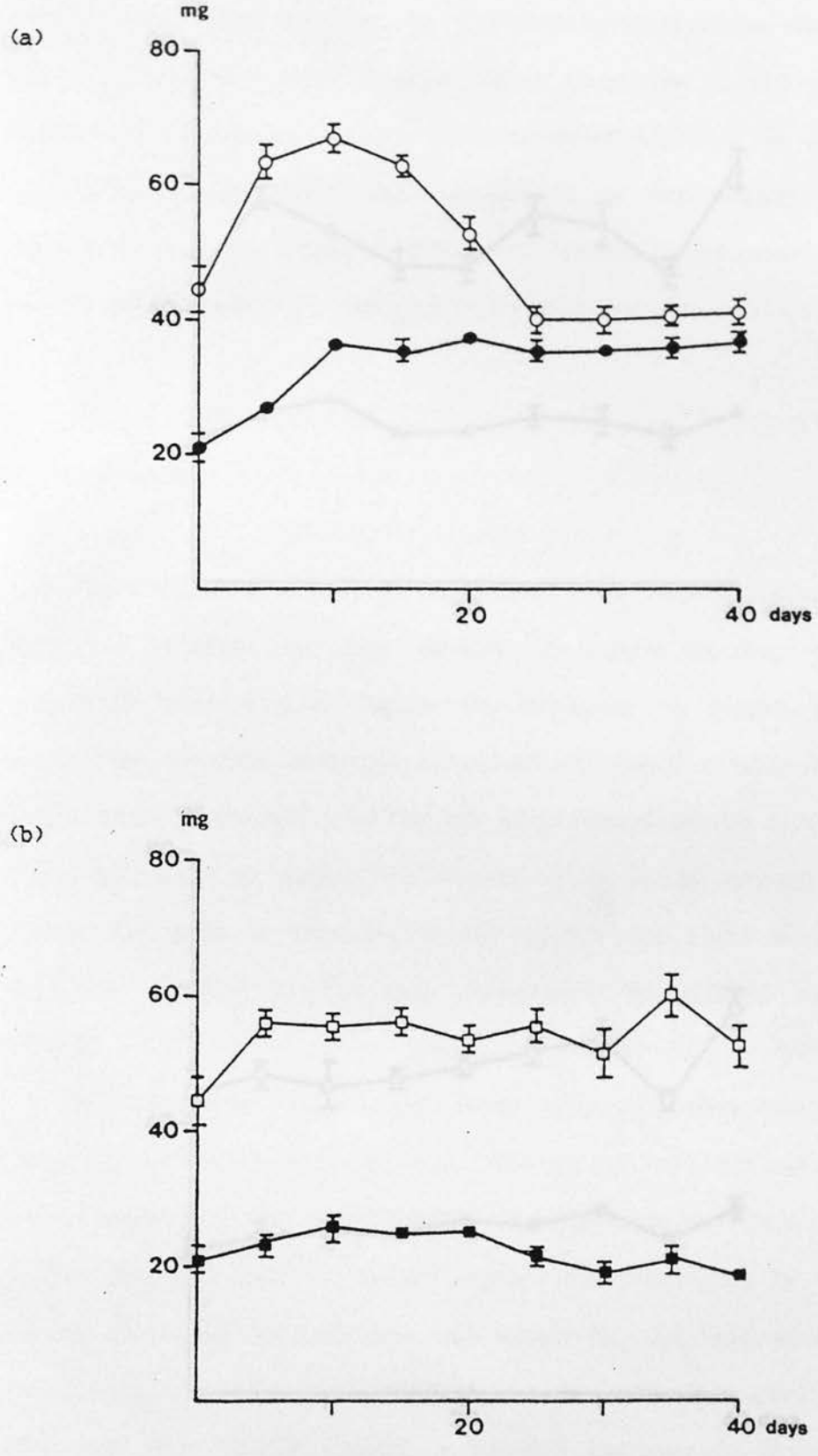


Fig. 3.3. Fresh (open symbols) and dry weights (closed symbols) of caryopses (a) from intact plants and (b) cultured on 6% sucrose medium in the second experiment. (contd. over).

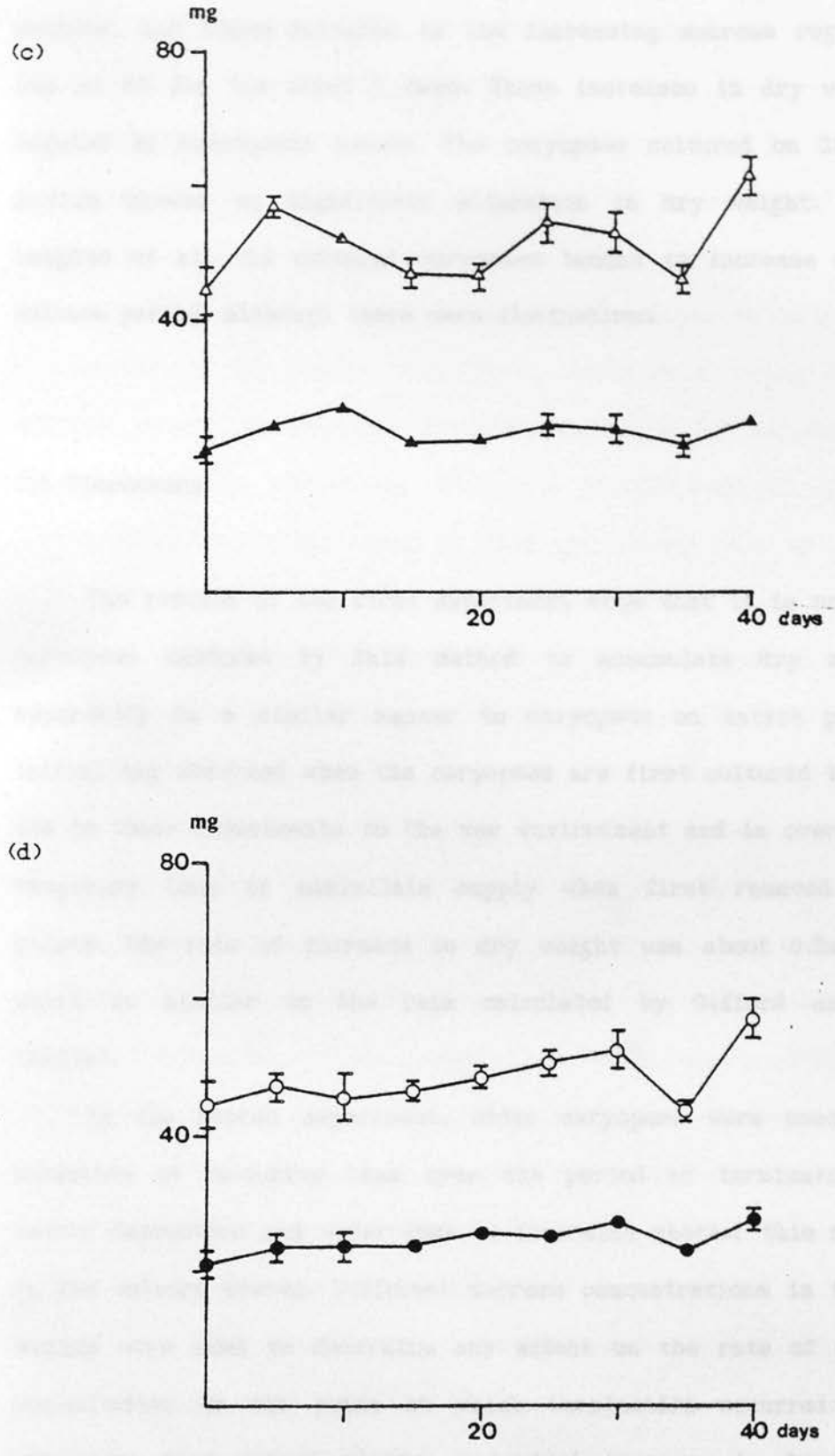


Fig. 3.3. Fresh and dry weights (symbols as before) of caryopses (c) cultured on increasing (6-18%) sucrose medium and (d) cultured on 18% sucrose medium in the second experiment.

sucrose, and those cultured in the increasing sucrose regime, which was at 6% for the first 7 days. These increases in dry weight were negated by subsequent losses. The caryopses cultured on 18% sucrose medium showed no significant alteration in dry weight. The fresh weights of all the cultured caryopses tended to increase during the culture period, although there were fluctuations.

3.4 Discussion.

The results of the first experiment show that it is possible for caryopses cultured by this method to accumulate dry matter and apparently in a similar manner to caryopses on intact plants. The initial lag observed when the caryopses are first cultured is possibly due to their adjustments to the new environment and in overcoming the temporary loss of assimilate supply when first removed from the plants. The rate of increase in dry weight was about 0.5mg per day which is similar to the rate calculated by Gifford and Bremner (1981a).

In the second experiment, older caryopses were used with the intention of culturing them over the period of termination of dry matter deposition and water loss to determine whether this could occur in the culture system. Different sucrose concentrations in the culture medium were used to determine any effect on the rate of dry matter accumulation or the point at which termination occurred. With the caryopses from intact plants, a typical increase in fresh and dry weights for the first 10 days of the experiment was seen. At this point they had reached 40 "days" on the developmental scale, so dry

weight was maintained while fresh weight declined, as expected from the results described in Chapter 2.

The lack of significant increase in dry weight in the cultured caryopses at any sucrose concentration could be explained by a lag phase similar to that observed in the first experiment. This could have taken up most of the available time between culturing and the termination of dry matter deposition, therefore allowing little time for dry weight to increase. However, there was no decline in fresh weight generally associated with the termination of dry matter accumulation, and after about 20 days in culture many of the grains started to assume a swollen appearance with fluid contents. It seems that the development of these caryopses was abnormal. The only difference observed between the sucrose concentrations was that the higher levels almost entirely inhibited precocious germination, which became a problem at the 6% sucrose level. This agrees with the work of Bottacin *et al.* (1985) and Craig (1983) with barley caryopses in that no optimum sucrose concentration for growth was found although the lack of any dry weight increase makes the correlation somewhat tenuous. The decline in dry weight observed in caryopses cultured on the 6% medium was probably due to mobilisation of reserves to fuel germination. The presence of swollen caryopses with fluid contents could be due to the hydrolysis of starch and / or to the uptake of medium without conversion to reserves.

These results suggest that caryopsis culture over short periods by this method would possibly be successful with caryopses early in development. However, the necessity for the caryopses to be at least partially submerged in the medium, and the probable high humidity within the plates prevents maturation. Since it seemed that this

technique was unlikely to prove successful in the study of termination of grain growth, at least without considerable inputs into development, it was not used in any subsequent work described here.

4. PHOTOSYNTHETIC AND RESPIRATORY ACTIVITY DURING DEVELOPMENT.

4.1 Introduction.

The contribution of pericarp photosynthesis to grain filling has been briefly discussed in Chapter 1. Nutbeam and Duffus (1978) used an oxygen electrode to measure light-dependent oxygen evolution and found that this increased with removal of the husk, and again if the pericarp transparent layer was removed for cultivars of wheat and barley at 25 days after anthesis. Similarly, oxygen uptake in the dark was also increased by removal of the husk and the transparent layer. They concluded that the outer layers formed a barrier to oxygen exchange. Watson (1985) found that caryopses of wheat and barley are capable of fixing externally-supplied ^{14}C-labelled carbon dioxide in the pericarp green layer and this too increased if the transparent layer was removed. It was concluded that the transparent layer is a barrier to carbon dioxide uptake.

The apparently low permeability of the transparent layer to oxygen and carbon dioxide has given rise to the theory that there might be cycling of these gases between photosynthesis and respiration within the caryopsis. Similar refixation of carbon dioxide respired by seeds has been found in legume pods (Flinn, Atkins and Pate, 1977). The discovery of high levels of phosphoenol pyruvate carboxylase (PEP carboxylase) in the pericarp green layer of barley and wheat implicated this enzyme in the refixation process (Duffus and Rosie, 1973a; Wirth, Kelly, Fischbeck and Latzko, 1977). Watson (1985) labelled barley endosperms using ^{14}C-sucrose by detached ear culture, and then looked at the ^{14}C content of the caryopsis after incubation

in the light or dark. More label was found to be present in the caryopsis after incubation in the light and it was concluded that the pericarp might therefore be re-fixing some of the $^{14}CO_2$ respired by the endosperm. Kriedemann (1966) investigated the re-fixation of respired carbon dioxide in whole ears by measuring CO_2 evolution in the light and dark of ears in a CO_2-free environment, and found that evolution was lower in the light, suggesting that refixation was occurring. However, in this case respiration and refixation could have been occurring in any part of the ear.

Rates of photosynthesis and respiration in wheat ears and flag leaves were studied throughout development by Evans and Rawson (1970). Their work included measurements for isolated caryopses using infra-red gas analysis to monitor carbon dioxide exchange. High rates of dark respiration were observed, particularly in the early stages of development. However, net photosynthesis in the light was not found at any stage, although photosynthesis did almost compensate for the observed rate of dark respiration in the first 20 days after anthesis. This suggests that this technique is less accurate than the oxygen electrode method of Nutbeam and Duffus (1978).

Some enzymes involved in photosynthesis have been studied in the pericarp by several groups (Duffus and Rosie, 1973a; Nutbeam and Duffus, 1976; Wirth *et al.*, 1977; Nutbeam, 1978). The high levels of PEP carboxylase found by these workers, combined with the high amounts of ^{14}C found in the C_4-acid, malic acid, one minute after the pericarp had been fed $^{14}CO_2$ (Nutbeam and Duffus, 1976), suggests that C_4 photosynthesis is the predominant pathway in the pericarp. Nutbeam (1978) found that activity of ribulose bisphosphate carboxylase, the enzyme involved in CO_2 fixation in the C_3 pathway, was considerably lower than that of PEP carboxylase, the corresponding enzyme in the C_4

pathway. Activity of PEP carboxylase in the barley pericarp was shown to parallel changes in chlorophyll content and was maximal at 25 days after anthesis. Although Evans and Rawson (1970) found that net photosynthesis of wheat grains was highest around 15 to 20 days after anthesis, this is probably around the period of maximum PEP carboxylase activity when differences between wheat and barley and the shorter growth period in the Australian climate are taken into account.

In contrast, the maximum rate of dark respiration was found by Evans and Rawson (1970) to be at about 15 days after anthesis which is somewhat earlier than the highest rate of certain respiratory enzyme activities, even taking into account climatic differences. Duffus and Rosie (1977) compared activities of enzymes from the glycolytic pathway and the pentose phosphate pathway in barley endosperms. They found that whereas glycolytic enzymes all showed maximum activity at 35 days after anthesis and then declined rapidly, the pentose phosphate pathway enzymes reached maximum activity at 30-35 days after anthesis which was then maintained until maturity. This suggested that this pathway might be the more important in the later stages of development. It is possible that the difference in timing of peak activity compared to dark respiration measured by Evans and Rawson (1970) might be due to differences between the endosperms used here and whole grains, or indeed between wheat and barley. However, Sangwan, Popli and Singh (1983) measured the activities of some glycolytic and pentose phosphate pathway enzymes in whole wheat grains and obtained a similar pattern of results although there was more of a decline in the activities of the pentose phosphate pathway enzymes during maturation. These workers also investigated the activities of two tricarboxylic acid cycle enzymes and obtained

results showing very similar patterns to those of the glycolytic enzymes.

The aim of the work described in this chapter is to compare patterns of photosynthesis and respiration throughout the development of the caryopsis with the patterns of dry matter deposition observed in Chapter 2 to see if these might be important in the termination of grain growth. Rates of photosynthesis and respiration were obtained by measuring oxygen exchange using the oxygen electrode technique of Nutbeam and Duffus (1978). Dichlorophenyl dimethylurea (DCMU), which was used as a spray by King, Wardlaw and Evans (1967) to inhibit ear photosynthesis, was used in this work to see if there was any evidence for photorespiration in the pericarp. Chlorophyll levels in the pericarp and the activities of some respiratory enzymes in the endosperm were also measured for comparison with the pattern of oxygen exchange throughout development of the caryopsis. Of the three respiratory enzymes studied, two are involved in the tricarboxylic acid cycle and one is a member of the electron transport chain. Malate dehydrogenase (E.C. 1.1.1.37) reversibly catalyses the formation of oxaloacetate from malate with reduction of the NAD^+ cofactor. Fumarase or fumarate hydratase (E.C. 4.2.1.2) reversibly catalyses the hydration of fumarate to malate. It is cytochrome oxidase (E.C. 1.9.3.1) that is a member of the electron transport chain. It is also known as cytochrome a/a_3 and is located across the mitochondrial membrane where it catalyses the oxidation of cytochrome C by molecular oxygen.

4.2 Materials and Methods.

4.2.1 Plant Material.

Wheat plants, *Triticum aestivum* cv. Sicco, were grown in the glasshouse, and aged as described in Section 2.2.1.

4.2.2 Chlorophyll Determination.

Chlorophyll was determined by the method of Wintermans and De Mots (1965). Pericarp green layers (10) were dissected from caryopses, homogenised in 1-3ml 96% ethanol at 70°C, and the debris spun down in a bench centrifuge. The supernatant was retained and the pellet re-extracted. The combined supernatants were diluted to a suitable volume (5 or 10ml) with ethanol, and absorbances at 649nm, 654nm and 665nm against an ethanol blank were recorded on a Pye Unicam SP8-100 UV/Vis spectrophotometer. Equations for the calculation of chlorophyll a, chlorophyll b and chlorophyll a+b, as derived from data in Wintermans and De Mots paper, are shown below:

Chlorophyll a = $13.70\ A_{665} - 5.76\ A_{649}$ µg/ml.

Chlorophyll b = $25.80\ A_{649} - 7.60\ A_{665}$ µg/ml.

Chlorophyll a+b = $1000\ A_{654} / 39.8$ µg/ml.

Controls in which the pericarp transparent layer was extracted in place of the green layer were included.

4.2.3 Respiratory Enzyme Assays.

The enzymes were prepared by homogenising endosperms in ice-cold 50mM MOPS buffer pH 7.0 in a hand-held all-glass homogeniser, and centrifuging in a swing-out rotor at 10000g for 15 min at 0-4°C. The supernatant was used for the assays.

Cytochrome C oxidase activity was measured by the method of Cooperstein and Lazarow (1951). The reaction mixture contained 1.7 x 10^{-5}M cytochrome C in 50mM MOPS pH7.0 which was reduced by addition of an aliquot of 1.2M sodium hydrosulphite freshly prepared (300µl per 100ml cytochrome C solution) and shaken vigorously to remove excess hydrosulphite. From this preparation, 1ml was mixed in a cuvette with 0.1ml enzyme preparation and oxidation of reduced cytochrome C was followed at 550nm on a Pye Unicam SP8-100 UV/Vis spectrophotometer against a blank identical except for the omission of cytochrome C from the reaction mixture. The amount of oxidised cytochrome C produced was calculated by the method of Cooperstein, Lazarow and Kurfess (1950). The equation used is shown below:

Δ[ox. Cyt. C] = slope / 1.96×10^4 mol min^{-1}.

where 1.96×10^4 is the difference between the molar extinction coefficients of oxidized and reduced cytochrome C.

Fumarase was assayed by the method of Pierpoint (1960), which in turn is a modification of the method of Racker (1950). A 0.1ml sample of the enzyme preparation was added to 1ml reaction mixture, which contained 50mM MOPS buffer pH7.0, 0.05M L-malic acid and 0.0007M L-cysteine. Production of fumarate was followed by the increase in optical density at 240nm against a blank which was identical except

that malic acid was omitted from the reaction mixture. A standard curve of fumarate concentration was used to calibrate the results.

Malate dehydrogenase was assayed by the method of Kornberg and Beevers (1957). The enzyme was assayed in the direction of malate production from oxaloacetate by adding 0.1ml enzyme preparation to a reaction mixture containing 50mM MOPS pH7.0, 25.9μM NADH and 1.034mM $MgCl_2$, and then adding 0.2ml 5mM oxaloacetate. Oxidation of NADH was followed by the decrease in optical density at 340nm against a blank identical except that NADH was omitted from the reaction mixture. A standard curve of NADH concentration was prepared for calibration of the results.

4.2.4 Measurement of Oxygen Exchange.

Rates of oxygen exchange were recorded using a Rank oyygen electrode by the method of Nutbeam and Duffus (1978). The chamber of the electrode contained 4.5ml 50mM tricine buffer (containing 1mM $MgCl_2$, 1mM $MnCl_2$ and 330mM sorbitol, pH7.5 with KOH) stirred at a constant speed with a magnetic follower, and jacketed by a circulating water supply at 25°C. Measurements were made in the light, with the chamber illuminated by a 275W Thorn EMI P1/1 photographic lamp at a distance of 15cm from the centre of the chamber giving a photon flux density of 700μE m^{-2} s^{-1} at the chamber, and in the dark using an aluminium foil shade. The effect of presence or absence of the photosynthetic inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU 1.33μM) was also studied. In preliminary trials not reported here, it was found that concentrations of DCMU above that used here did not give greater inhibition of oxygen evolution. Oxygen exchange was recorded for whole caryopses, caryopses with the pericarp transparent

layer removed and endosperms, using three per measurement. Control runs, in which no tissue was included, were performed after every three measurements. The results were calibrated by a Winkler oxygen titration (Golterman, Clymo and Ohnstad, 1978) in which oxygen combines with manganous hydroxide to form higher hydroxides. When acidified in the presence of iodide, these hydroxides liberate iodine in amounts equivalent to the original oxygen content and this is determined by titration with sodium thiosulphate.

4.3 Results.

4.3.1 Chlorophyll Content.

The results presented in Fig. 4.1a show changes in chlorophyll content of the pericarp green layer during development. The results in Fig. 4.1b are chlorophyll contents from field-grown material, the same material as was used for the analyses presented in Chapter 2, and is included here as a further comparison between glasshouse and field-grown material. In both situations, levels of chlorophyll a exceeded those of chlorophyll b throughout development, except in the very late stages of development of field material when total chlorophyll levels were very low.

Maximum total chlorophyll was achieved at 20 "days" after anthesis and this was maintained until 25 "days" for field-grown material before starting to decline sharply. In glasshouse-grown material, the decline started immediately at 20 "days", but was more gradual to start with. The mean maximum level was similar for glasshouse and field samples. Levels of almost zero were reached at 45 "days" for glasshouse, and 50 "days" for field samples. Levels of

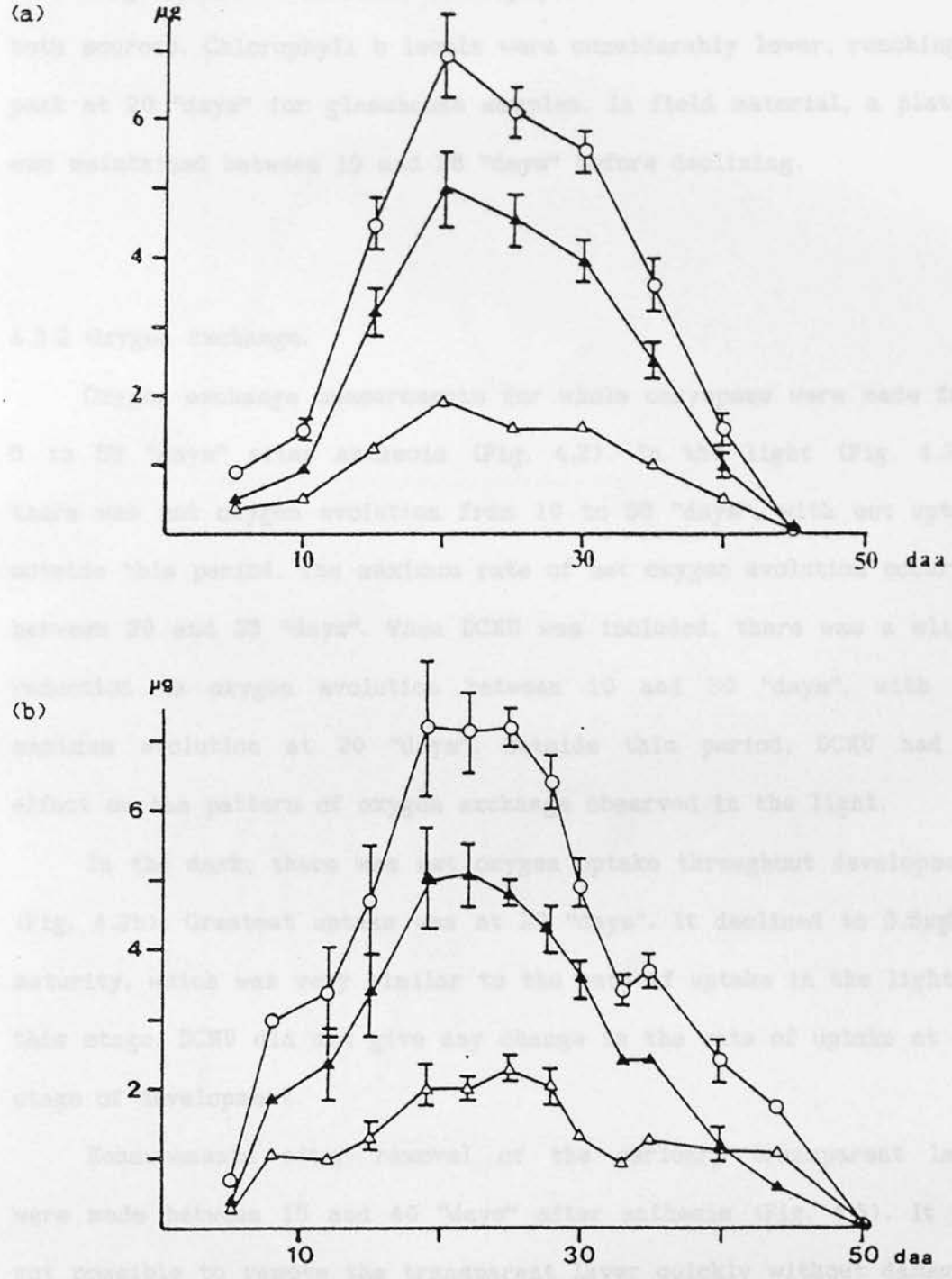


Fig. 4.1. Levels in the pericarp green layer of total chlorophyll (O), chlorophyll a (▲) and chlorophyll b (Δ) of (a) glasshouse-grown caryopses and (b) field-grown caryopses.

chlorophyll a followed the same pattern as for total chlorophyll, but were slightly lower. Maximum chlorophyll a content was the same from both sources. Chlorophyll b levels were considerably lower, reaching a peak at 20 "days" for glasshouse samples. In field material, a plateau was maintained between 19 and 28 "days" before declining.

4.3.2 Oxygen Exchange.

Oxygen exchange measurements for whole caryopses were made from 5 to 55 "days" after anthesis (Fig. 4.2). In the light (Fig. 4.2a), there was net oxygen evolution from 10 to 35 "days", with net uptake outside this period. The maximum rate of net oxygen evolution occurred between 20 and 25 "days". When DCMU was included, there was a slight reduction in oxygen evolution between 10 and 30 "days", with the maximum evolution at 20 "days". Outside this period, DCMU had no effect on the pattern of oxygen exchange observed in the light.

In the dark, there was net oxygen uptake throughout development. (Fig. 4.2b). Greatest uptake was at 20 "days". It declined to 3.5$\mu g h^{-1}$ at maturity, which was very similar to the rate of uptake in the light at this stage. DCMU did not give any change in the rate of uptake at any stage of development.

Measurements after removal of the pericarp transparent layer were made between 15 and 40 "days" after anthesis (Fig. 4.3). It was not possible to remove the transparent layer quickly without damaging the remaining tissues at other ages. In the light (Fig. 4.3a), there was net oxygen evolution from 15 to 25 "days". This was at a fairly constant rate during this period, after which it declined, giving net uptake after 30 "days". When DCMU was included, there was net oxygen uptake throughout the period of measurement which was maximal at 15

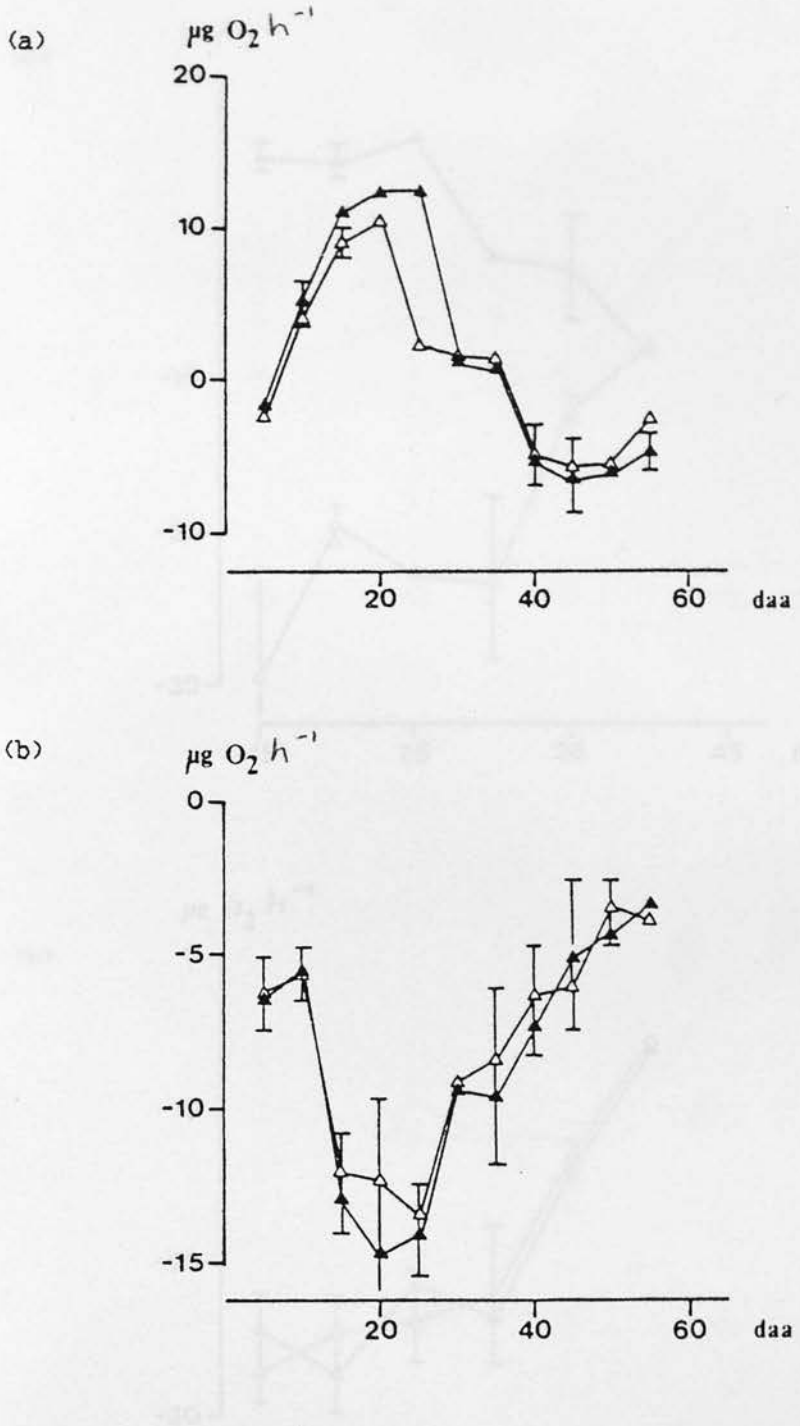


Fig. 4.2. Oxygen evolution per intact caryopsis in the presence (△) and absence (▲) of DCMU (a) in the light and (b) in the dark.

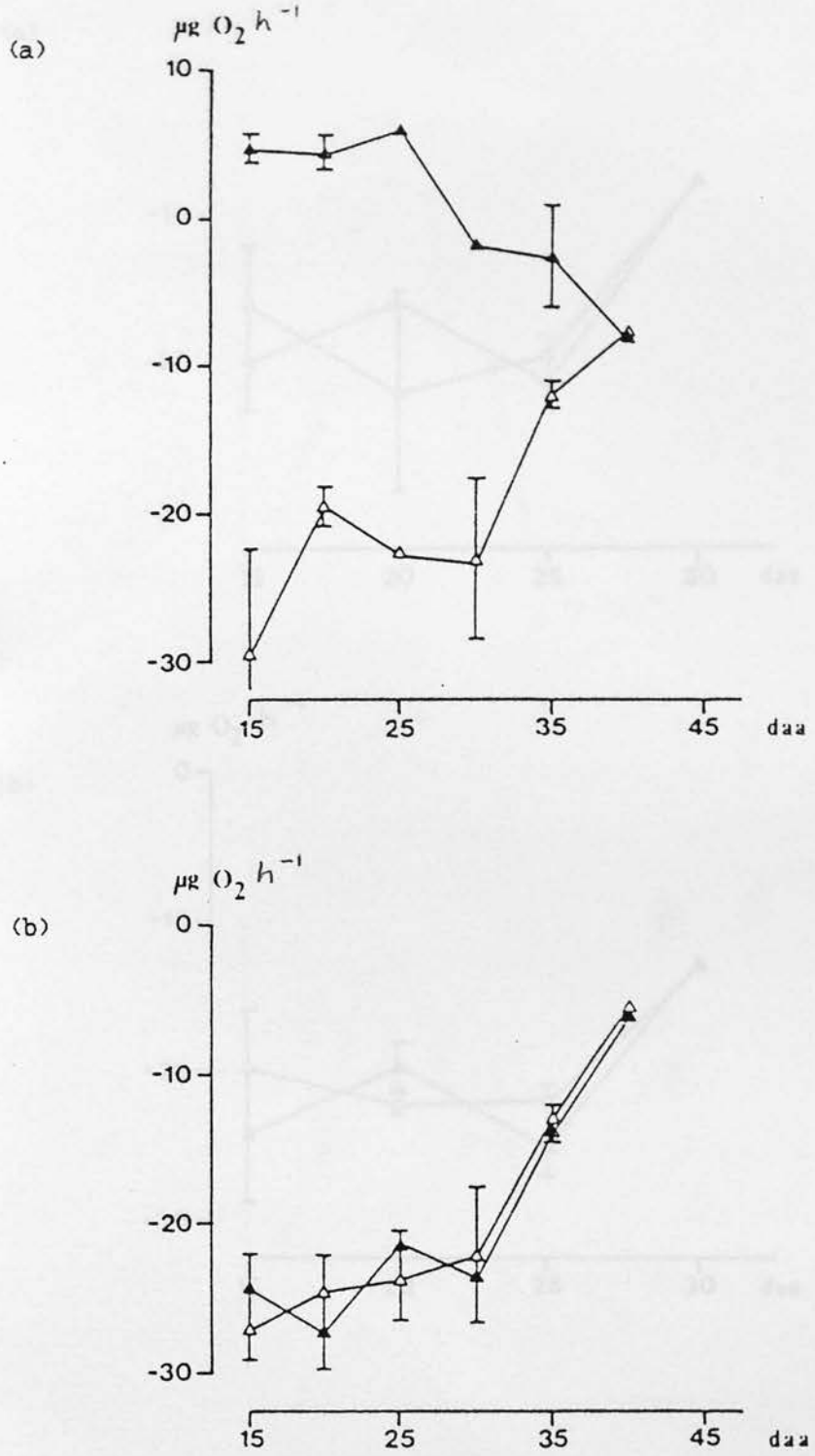


Fig. 4.3. Oxygen evolution per caryopsis with the pericarp transparent layer removed in the presence (△) and absence (▲) of DCMU (a) in the light and (b) in the dark.

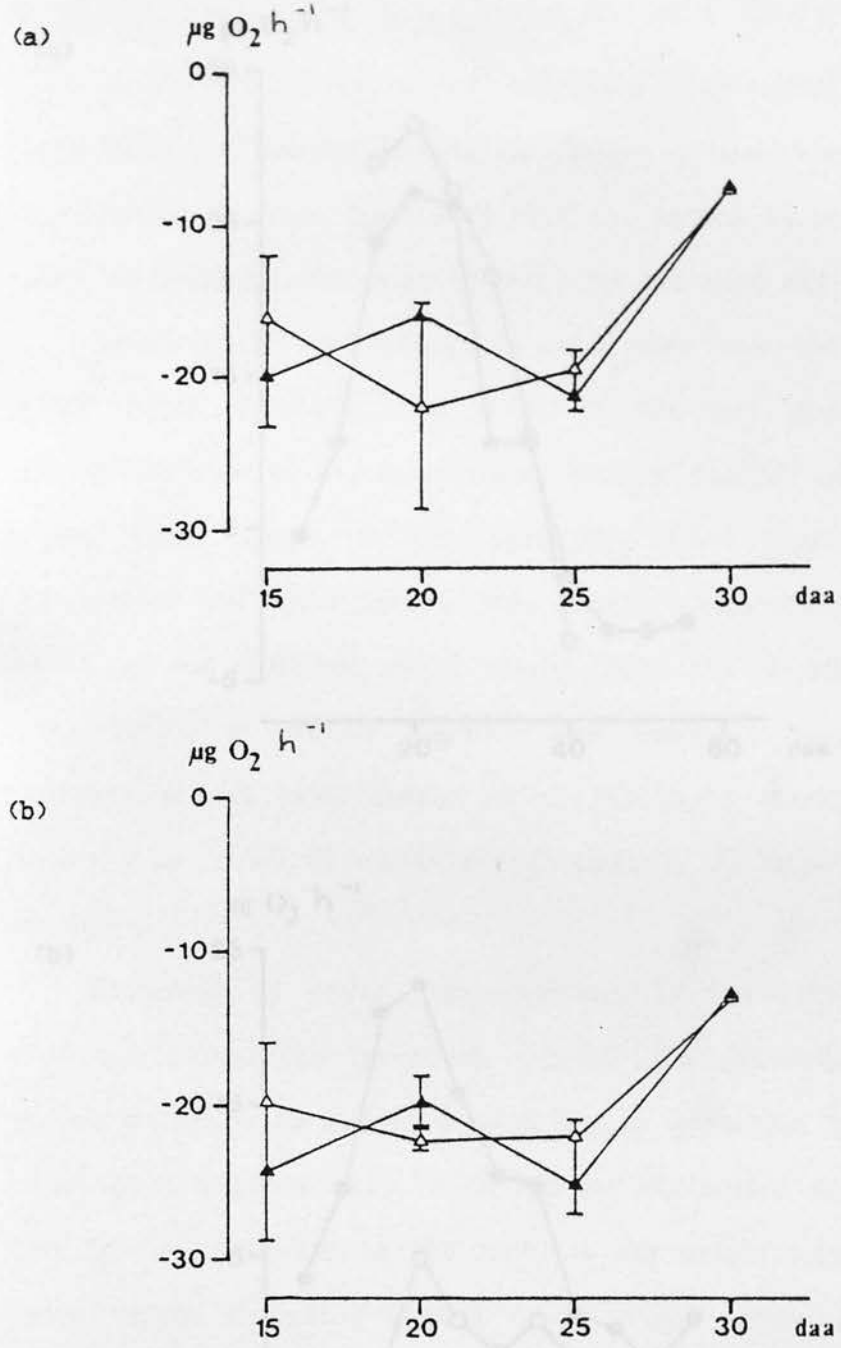


Fig. 4.4. Oxygen evolution per endosperm in the presence (△) and absence (▲) of DCMU (a) in the light and (b) in the dark.

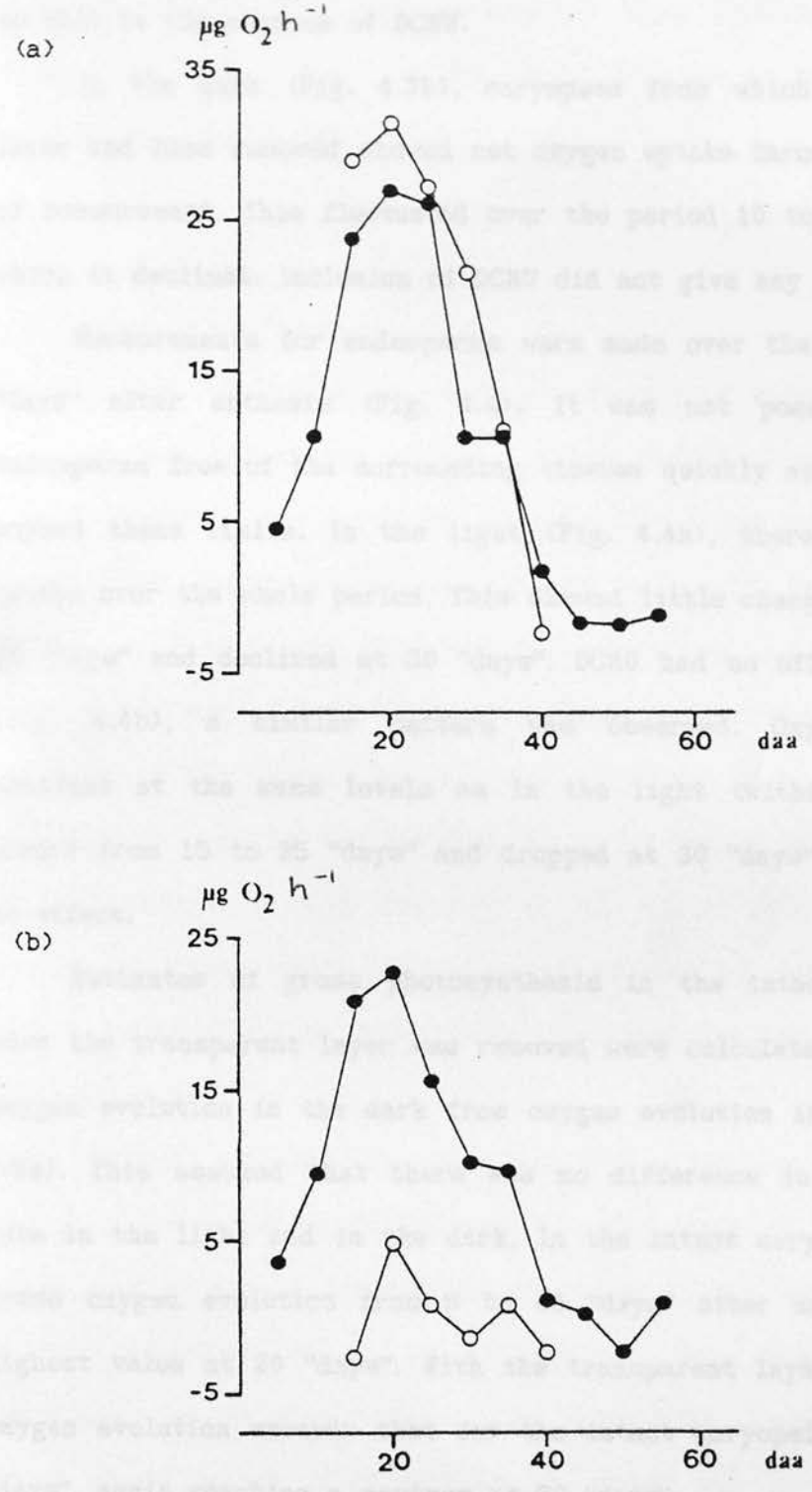


Fig. 4.5. (a) Gross oxygen evolution and (b) the difference between oxygen evolution in the dark and in the light in the presence of DCMU in intact caryopses (●) and caryopses with the pericarp transparent layer removed (○).

"days" and then declined. At 40 "days" the rate of uptake was similar to that in the absence of DCMU.

In the dark (Fig. 4.3b), caryopses from which the transparent layer had been removed showed net oxygen uptake throughout the period of measurement. This fluctuated over the period 15 to 30 "days", after which it declined. Inclusion of DCMU did not give any change.

Measurements for endosperms were made over the period 15 to 30 "days" after anthesis (Fig. 4.4). It was not possible to dissect endosperms free of the surrounding tissues quickly and without damage beyond these limits. In the light (Fig. 4.4a), there was net oxygen uptake over the whole period. This showed little change between 15 and 25 "days" and declined at 30 "days". DCMU had no effect. In the dark (Fig. 4.4b), a similar pattern was observed. Oxygen uptake was constant at the same levels as in the light (within the limits of error) from 15 to 25 "days" and dropped at 30 "days". DCMU again had no effect.

Estimates of gross photosynthesis in the intact caryopsis and when the transparent layer was removed were calculated by subtracting oxygen evolution in the dark from oxygen evolution in the light (Fig. 4.5a). This assumed that there was no difference in the respiratory rate in the light and in the dark. In the intact caryopsis, there was gross oxygen evolution from 5 to 40 "days" after anthesis with the highest value at 20 "days". With the transparent layer removed, gross oxygen evolution exceeds that for the intact caryopsis from 15 to 30 "days", again reaching a maximum at 20 "days".

Evidence for photorespiration in intact caryopses and when the transparent layer was removed was calculated as the difference in the rate of oxygen uptake between the dark rate and the light rate when DCMU was included (Fig. 4.5b). With intact caryopses, the results

showed oxygen uptake only at 50 "days" after anthesis, and only at very low levels. With the transparent layer removed, there was net oxygen uptake at 15, 30 and 40 "days", but not in between, and again only at very low levels.

4.3.3 Respiratory Enzymes.

Results for the measurement of activity of malate dehydrogenase, fumarase and cytochrome oxidase in the endosperm are are presented both as activity per endosperm and as activity per mg dry weight.

Malate dehydrogenase showed an initially high activity per endosperm at 20 "days" after anthesis which was followed by a drop at 25 "days" (Fig. 4.6a). There was then an increase to a maximum activity of 152 nkatals at 35 "days". A slight drop to 40 "days" was followed by a sharp decline from 45 to 50 "days". When expressed per mg dry weight, a high activity at 20 "days" which dropped by 25 "days" was found (Fig. 4.6b). Between 25 and 35 "days" activity was fairly constant and it then showed a steady decline to low levels at 45 to 50 "days".

Fumarase activity per endosperm (Fig. 4.7a) was constant from 15 to 20 "days" after anthesis. Activity then fell at 25 "days" before increasing to a peak activity at 40 "days". There was a sharp decline to low activities at 45 to 50 "days". When these activities are expressed per mg dry weight (Fig. 4.7b), the highest activity was found at 15 "days". This fell at 25 "days" and then was maintained until 40 "days." It then dropped again to become almost zero from 45 "days".

Activity of cytochrome oxidase per endosperm (Fig. 4.8a) was comparatively low to start with. Between 15 and 25 "days" after

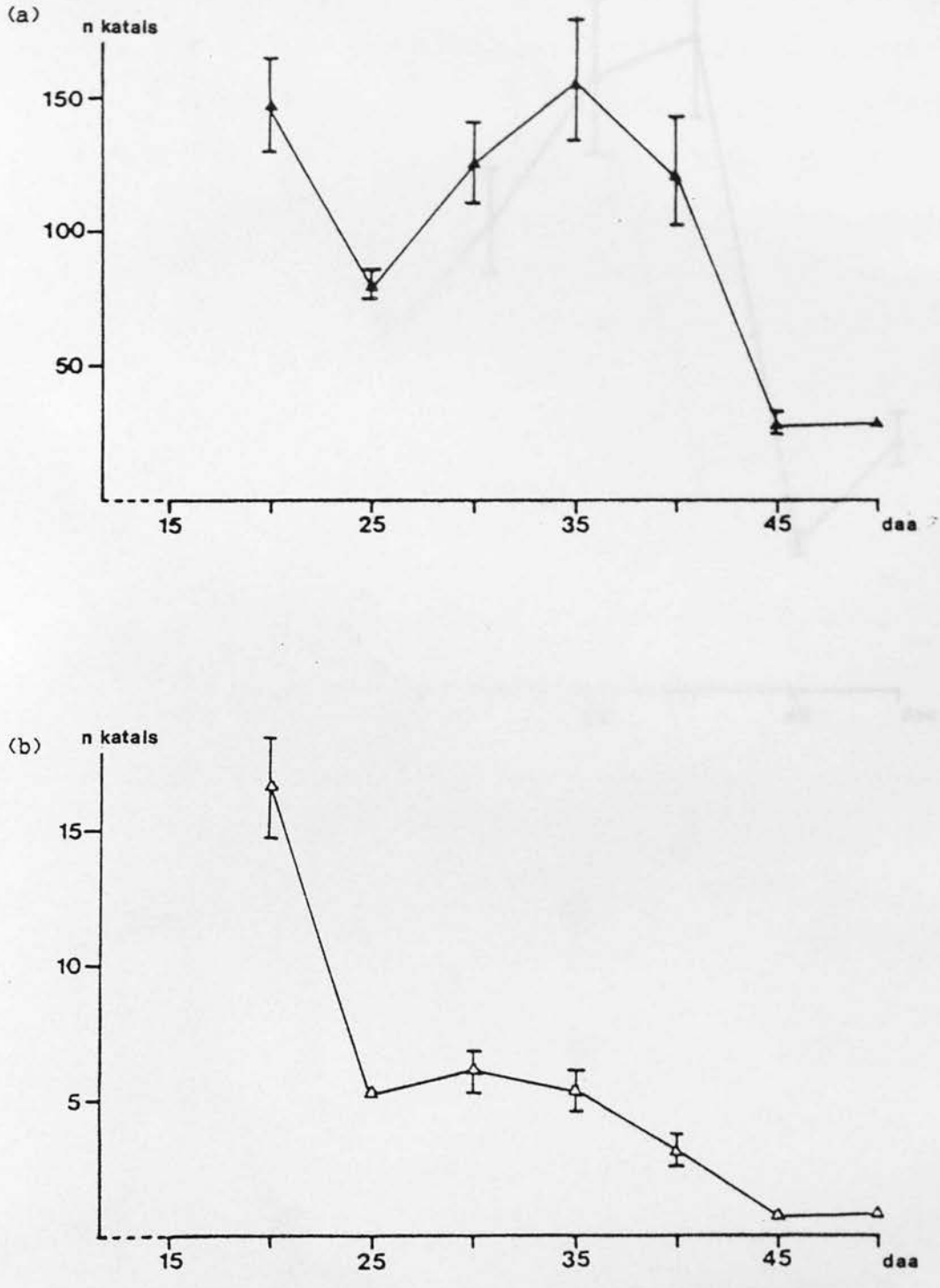


Fig. 4.6. Activity of malate dehydrogenase (a) per endosperm and (b) per mg endosperm dry weight.

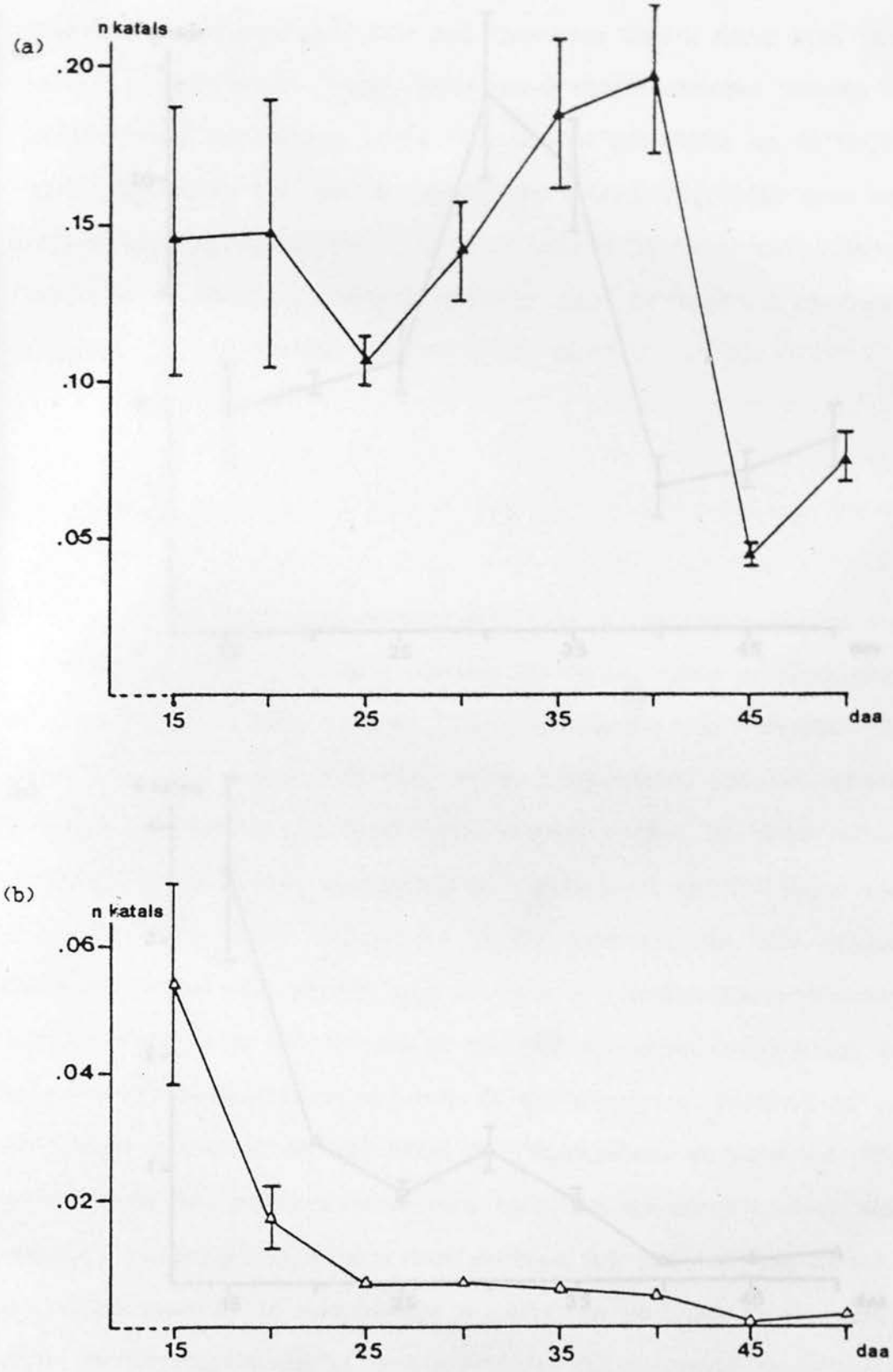


Fig. 4.7. Activity of fumarase (a) per endosperm and (b) per mg endosperm dry weight.

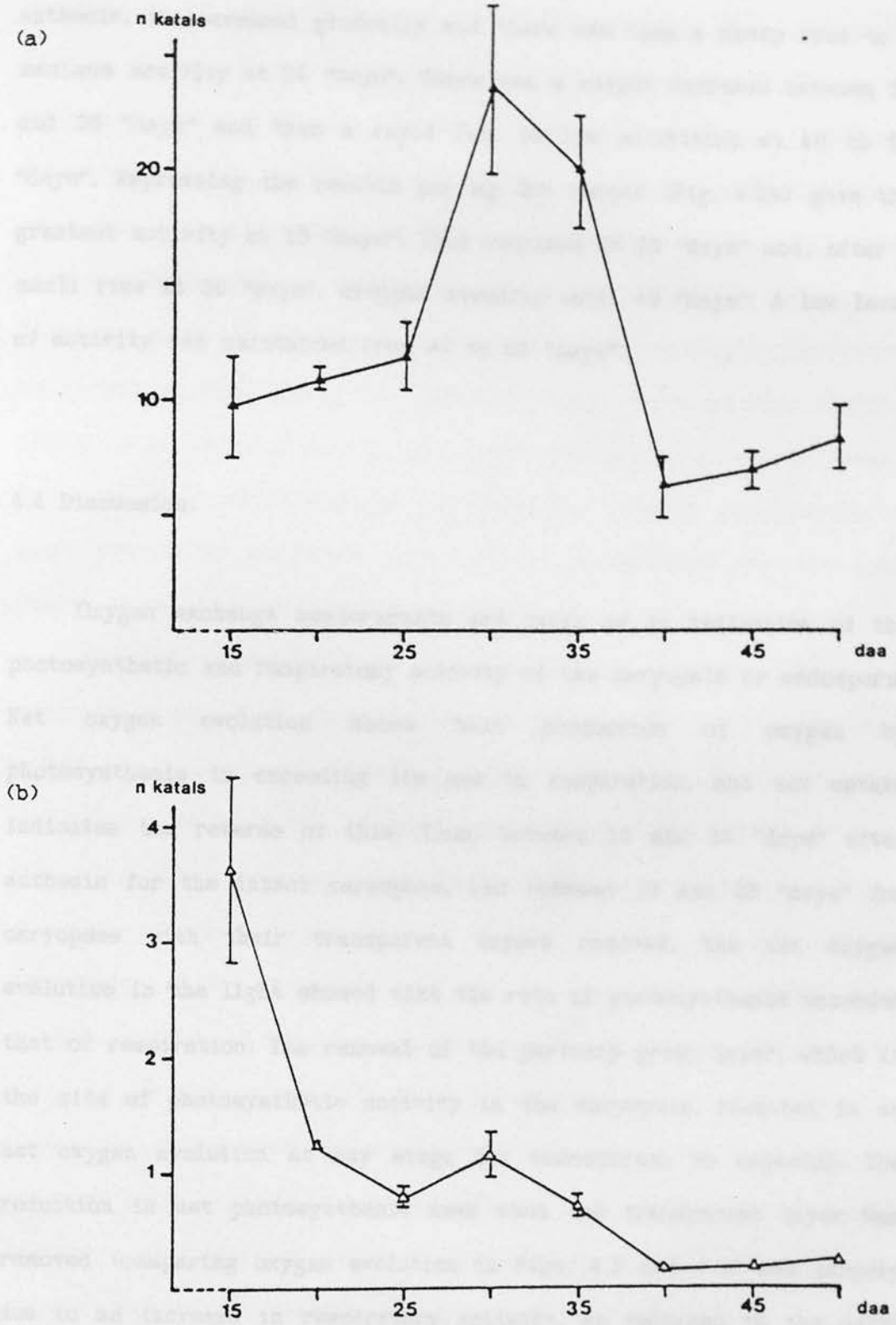


Fig. 4.8. Activity of cytochrome C oxidase (a) per endosperm and (b) per mg endosperm dry weight.

anthesis, it increased gradually and there was then a sharp rise to a maximum activity at 30 "days". There was a slight decrease between 30 and 35 "days" and then a rapid fall to low activities at 40 to 50 "days". Expressing the results per mg dry weight (Fig. 4.8b) gave the greatest activity at 15 "days". This declined at 25 "days" and, after a small rise at 30 "days", dropped steadily until 40 "days". A low level of activity was maintained from 40 to 50 "days".

4.4 Discussion.

Oxygen exchange measurements are taken as an indication of the photosynthetic and respiratory activity of the caryopsis or endosperm. Net oxygen evolution shows that production of oxygen by photosynthesis is exceeding its use in respiration, and net uptake indicates the reverse of this. Thus, between 10 and 30 "days" after anthesis for the intact caryopses, and between 15 and 25 "days" for caryopses with their transparent layers removed, the net oxygen evolution in the light showed that the rate of photosynthesis exceeded that of respiration. The removal of the pericarp green layer, which is the site of photosynthetic activity in the caryopsis, resulted in no net oxygen evolution at any stage for endosperms, as expected. The reduction in net photosynthesis seen when the transparent layer was removed (comparing oxygen evolution in Figs. 4.2 and 4.3) was largely due to an increase in respiratory activity, as recorded in the dark. Gross photosynthesis was in fact greater in the caryopses minus their transparent layers (Fig. 4.5a).

Oxygen uptake in the dark is a measure of the respiratory activity of the caryopsis. Respiratory activity was highest in

caryopses with or without the transparent layer and endosperms between 15 and 25 "days" after anthesis. The fact that activity in the caryopsis was considerably greater when the transparent layer was removed suggests that this layer is limiting oxygen uptake, and is in agreement with the results of Nutbeam and Duffus (1978). That gross photosynthesis (oxygen evolution) was higher when the transparent layer was removed may also indicate reduced oxygen efflux, limitations on carbon dioxide uptake restricting photosynthetic activity (Watson, 1985), or a combination of both these factors when it is present. Rates of dark respiration of the caryopsis without the transparent layer and of the endosperm were similar, so it seems that the other layers of the caryopsis do not form a barrier to oxygen exchange.

The low permeability of the transparent layer to oxygen and carbon dioxide raises again the possibility of cycling of these gases within the caryopsis (Nutbeam and Duffus, 1976; Wirth, Kelly, Fischbeck and Latzko, 1977; Duffus, Nutbeam and Scragg, 1984). It is possible that when the transparent layer is in place, some of the requirement of pericarp photosynthesis for carbon dioxide is supplied by respiration of the caryopsis, and some of the oxygen for respiration is the product of pericarp photosynthesis. Therefore, the measured rates of exchange give activities lower than is actually the case. When the transparent layer is removed, the gases produced by photosynthesis and respiration are not retained in the confines of the caryopsis, but diffuse into the surrounding buffer. Thus, all gas requirements of respiration and photosynthesis have to be satisfied from the buffer, and there is an apparent increase in the activity of both processes.

DCMU is an inhibitor of electron flow in photosystem II of the photosynthetic electron transport chain. The inclusion of DCMU in the

buffer slightly inhibited photosynthesis in the intact caryopses, as is evidenced by a reduction in oxygen evolution. However, there was still oxygen evolution occurring which showed that photosynthesis was not totally inhibited. When the transparent layer was removed, there was a change from net oxygen evolution to net oxygen uptake when DCMU was included. The rate of oxygen uptake observed was very similar to that observed in the dark, suggesting that photosynthesis had been totally inhibited. The difference in the results with DCMU between caryopses with and without their transparent layers implies that the transparent layer is also a barrier to DCMU. Since DCMU is a photosynthetic inhibitor, it was expected that it would have no effect in the dark when photosynthesis does not take place, or in tissues from which the pericarp green layer had been removed and this was found to be the case.

Photorespiration is a catabolic process whereby oxygen is consumed and carbon dioxide evolved. Since the substrate is derived directly from ribulose bisphosphate as a consequence of the oxygenase activity of ribulose bisphosphate carboxylase / oxygenase from the Calvin cycle of photosynthesis, photorespiration is light-dependent. Evidence for photorespiration was sought by looking for differences in the rate of oxygen uptake in the dark and in the light when photosynthesis was inhibited by DCMU. Since photosynthesis was incompletely inhibited in the intact caryopses, the results still show oxygen evolution and it is impossible to tell whether photorespiration is occurring or not. With the transparent layer removed, the difference between dark uptake and light uptake in the presence of DCMU fluctuates between evolution and uptake. Where there is uptake, this could be taken as evidence of additional respiration, i.e. photorespiration. However, the amounts of uptake or evolution are very

small and are within the errors of the results from which they are calculated, and there is no consistent trend. Therefore, there is no solid evidence for photorespiration. This is perhaps consistent with the pericarp being a C_4 photosynthetic tissue since photorespiration is generally not easily detectable in C_4 plants. This may be due to refixation of the carbon dioxide it produces or because photorespiration is low or non-existent. A lack of photorespiration would be an advantage to the caryopsis as assimilate supplied would not be wasted by this pathway and could be used for synthesis of reserve material.

The level of total chlorophyll in the pericarp green layer is similar to that found in barley pericarps by Duffus and Rosie (1973a). The level found here for wheat was slightly higher than in barley, these workers finding a maximum of about 5μg per pericarp. It also reaches its highest level earlier than the barley which did not peak until 27 days after anthesis. The maximum chlorophyll level occurring at 20 "days" after anthesis correlates well with the maximum rate of gross photosynthesis as measured by oxygen exchange which also occurred at 20 "days". The similarity of the patterns of gross photosynthetic activity and chlorophyll content of the pericarp suggests that photosynthetic activity may be controlled by changes in the chlorophyll content. The ratio of chlorophyll a to chlorophyll b at the point of maximum total chlorophyll content is about 3.0. Several groups of workers (Black and Mayne, 1970; Chang and Troughton, 1972, Holden, 1973) have demonstrated that the chlorophyll a: b ratio is higher in C_4 than C_3 plant leaves, but a ratio of 3.0 is more consistent with results given for C_3 leaves. Thus, it appears that this is one factor against the pericarp being a C_4 tissue, although it

may just be a difference between the pericarp and leaves. A similarly low ratio was found in barley pericarps by Nutbeam (1978).

The activities of the three respiratory enzymes were expressed per endosperm and per mg dry weight to determine whether changes in activity per endosperm reflected changes in enzyme concentration and activity in the tissue or whether they were just sufficient to maintain a constant activity as the endosperm increased in size. They all showed similar patterns when expressed on a per mg dry weight basis. An initially high activity can be associated with the cell division phase of grain development, when the high levels of ATP generated by active respiration are needed as an energy source for cell division and the rapid synthesis of structural components. The period of fairly constant activity per mg dry weight seen for all three enzymes between 25 and 35 "days" can be associated with the enlargement and grain filling period, when a constant energy level might be required for the steady synthesis of storage products. The increase of activity per endosperm over this period can then be seen as that necessary to parallel dry weight increase and maintain a constant enzyme concentration in the cells. The final decline to low levels of activity probably indicates a general shut-down of metabolic activity as the grain dries out. The onset of this phase at about 35 "days" after anthesis for two of the enzymes, malate dehydrogenase and cytochrome oxidase, suggests that this might be one of the triggers for the termination of dry matter deposition, which was found to occur at 40 "days" after anthesis in Chapter 2. Measurement of respiratory activity in the endosperm by oxygen exchange showed a decline starting at 25 "days" after anthesis, suggesting that this process may start even earlier than the enzyme activities indicate.

Changes in activity per endosperm of the three respiratory enzymes are similar to the patterns of activity found for glycolytic and tricarboxylic acid cycle enzymes by Duffus and Rosie (1977) and Sangwan *et al.* (1983). However, these workers did not find the relatively high activities in early development seen here for malate dehydrogenase and fumarase in any of the enzymes they studied. The peak activities per endosperm of the three enzymes do not occur together, although overlapping of the error bars shows no significant change in malate dehydrogenase activity between 30 and 40 "days" and in fumarase activity from 35 to 40 "days". That cytochrome oxidase activity is highest at 30 "days" and then declines could indicate that respiratory activity starts to decrease earlier than at 35 or 40 "days" as indicated by the other two enzymes. The maintained activity of cytochrome oxidase is essential for respiration to continue. A decline in its activity correlates with the decline in oxygen uptake after 25 "days".

The large difference in the actual activies of malate dehydrogenase and fumarase is perhaps surprising since they are consecutive enzymes in the tricarboxylic acid cycle. The fact that malate dehydrogenase was, for convenience, measured in the reverse direction to that operating in the tricarboxylic acid cycle will have made a difference here. Fortunately, it is the changes in the patterns of activity rather than the absolute activities which are of interest.

When cytochrome oxidase catalyses the oxidation of cytochrome C, the stoichiometry of the reaction requires that four molecules of cytochrome C are oxidised per molecule of oxygen consumed. If the rate of oxygen uptake per endosperm at 25 "days" is converted, it gives a rate of 0.22nmol O_2 per second per endosperm, which has a requirement of 0.88nmol cytochrome C per second per endosperm, or 0.88 nkatals

cytochrome oxidase activity. The value for cytochrome oxidase activity measured here is considerably higher than this, which may indicate that the endosperm is capable of much higher respiratory activity than is actually occurring. A higher potential activity could explain the apparent discrepancy in the decline in endosperm respiratory activity which appears to commence at 25 "days" after anthesis when oxygen exchange was measured but not until later when enzyme activities were measured. However, it is also possible that the rate of respiration measured by oxygen exchange in whole endosperms is lower than the true value as there is not free circulation of gases exchanged in the centre of the tissue.

With reference to termination of dry matter deposition, photosynthesis and respiration present different potential sites for regulation. Gross photosynthesis, which it has been shown may be dependent on chlorophyll content, was highest at 20 "days" after which it declined. One possibility is that the decline of photosynthetic activity limits availability of sucrose, thus limiting dry matter accumulation and respiratory activity. However, this is unlikely since the results from Chapter 2 indicated that sucrose levels are maintained until after the termination of dry matter accumulation. That sucrose levels in the endosperm do not increase when starch synthesis ceases and respiration is declining indicates that there must be some regulation of sucrose entry. This could be related to the decline in photosynthesis, perhaps with reduced consumption matching lower supplies so that the overall level is unchanged.

The precise point at which respiratory activity starts to decline remains unclear. As has been shown, different measurements give different times at which the decline commences. However, all show a decline which starts before the termination of dry matter

accumulation. It could be that when respiratory activity drops below a critical level, synthesis of reserve material is unable to continue. For every molecule of oxygen used by cytochrome oxidase, 3 molecules of ATP are generated by oxidative phosphorylation. When each molecule of glucose is incorporated into starch, 2 molecules of ATP are consumed (Fig. 1.5). The levels of cytochrome oxidase activity per endosperm measured here can be calculated to produce amounts of ATP far in excess of the requirements of starch synthesis at the rates observed in Chapter 2. The ATP is also being used as an energy source for many other pathways in the cells. Thus, it is not possible to speculate on what levels of respiration would inhibit starch synthesis, although the levels of oxygen uptake measured may give an indication.

Termination of dry matter deposition as one stage in a programmed sequence of events is also a possibility. There is evidence here that such a sequence may be operating since decline in photosynthetic activity precedes decline in respiratory activity which in turn occurs before termination of dry matter accumulation.

5. COMPARISON OF STARCH DEPOSITION IN TWO CULTIVARS.

5.1 Introduction.

The final weight of an individual wheat grain is thought to be dependent on the number of cells in the endosperm, the rate of dry matter accumulation and its duration (Chapter 1). Starch contributes a large percentage of the final grain dry matter and the termination of its deposition is at the same point as the termination of grain growth (Chapter 2). Therefore, it seems likely that rate and duration of starch deposition are critical in determination of final grain weight. The aim of the work described in this chapter is to study some factors affecting starch accumulation.

Two cultivars which differ in mature grain weight were selected for this work. These were cv. Fenman, a winter wheat but one which can be grown as a spring wheat as its vernalisation requirement is very low and which has a high final grain weight, and cv. Broom, a spring wheat similar to cv. Sicco which has a low final grain weight. Differences in the pattern of starch deposition and assimilate supply (sucrose) between the two cultivars were determined during initial studies.

The aim of subsequent studies was to investigate whether elevated temperatures affected starch deposition in the two cultivars in the same way. Effects of elevated temperatures on grain-filling have been shown to include an increase in the rate of dry matter deposition, but a reduction in the duration (Chapter 1). Measurements of the activity of the enzyme starch synthase were included in this work to determine whether or not changes in starch levels observed

might be due to alterations in the capacity of the biosynthetic pathway. Measurements of both soluble and granule-bound forms of starch synthase, and their activities with both ADPG and UDPG were included to determine the relative importance of each form of the enzyme and each substrate to starch accumulation. With this approach, it was hoped to see whether there were any changes in the contribution of the two enzymes or substrates at the different temperatures and during grain filling.

5.2 Materials and Methods.

5.2.1 Plant Material.

Wheat plants of two cultivars, *Triticum aestivum* cv. Fenman and cv. Broom, were used. Glasshouse material was grown as described in Section 2.2.1. Stage of development was assessed using the scale for cv. Sicco, but omitting size data.

Growth room experiments were carried out in Fisons 1300 plant growth rooms. Individual plants were grown in 5cm pots in John Innes no. 2 compost. Irrigation was automatic to levels sufficient to prevent water stress, and they were fed twice weekly with Chempak (1.5g Chempak no. 1 and 0.75g Chempak no. 2 per litre of water, Chempak Products, Hertford). Light was supplied with 125W warm white fluorescent tubes and 60W incandescent bulbs giving a photon flux density at ear height of 825μE m^{-2} s^{-1}. The photoperiod was 18h, and relative humidity was maintained at 65%. Plants were grown from sowing until 10 days after anthesis in a 20/15°C day/night temperature regime. At this point, one third of the plants of each cultivar remained in this regime, and a third were transferred to

either 15/10°C or 30/25°C regimes. In this experiment, caryopses were not "aged" according to the developmental scale established with cv. Sicco. Age was recorded in actual days after anthesis (daa = days after anthesis for these results).

5.2.2 Fresh and Dry Weights.

Fresh and dry weights of caryopses and endosperms were measured as described in Section 2.2.2.

5.2.3 Sucrose, Starch and Nitrogen Determination.

Sucrose, starch and nitrogen levels were determined by the methods described in Sections 2.2.3, 2.2.4 and 2.2.5 respectively.

5.2.4 Extraction of Bound and Soluble Starch Synthase.

Bound and soluble starch synthases were extracted according to the method of Mengel and Judel (1981). Endosperms (10-12) were homogenised on ice in approximately 2ml ice-cold 50mM MOPS buffer pH7.7 containing 2mM EDTA and 1.3mM dithiothreitol. The homogenate was filtered through 3 layers of muslin, rinsing with 1ml buffer, and centrifuged at 5000g for 10 min at 0-4°C in a swing-out rotor. The supernatant was stored on ice and the pellet resuspended in 0.5-1ml buffer and recentrifuged. The final pellet suspended in 0.5-0.75ml buffer constituted the preparation of bound starch synthase.

Protein in the combined supernatants was precipitated by the addition of 85% ammonium sulphate, stirred at 2°C for 30 min and

centrifuged at 15000g for 15 min. The pellet suspended in a minimum volume of buffer was the soluble starch synthase preparation.

5.2.5 Assay of Starch Synthase Activity.

The activity of bound and soluble forms of starch synthase (ADP (UDP)-glucose: α-(1,4)-glucan α-4-glucosyl transferase E.C. 2.4.1.21 (E.C. 2.4.1.11 for the UDPG enzyme)) was assayed by a variation of the standard procedure (e.g. Leloir, de Fekete and Cardini, 1961; Baxter, 1972; Ozbun, Hawker and Preiss, 1972; Hawker, Marschner and Downton, 1974; MacDonald and Preiss, 1983). The reaction mixture comprised 0.075ml 166.7mM glycylglycine buffer pH8.3, 0.025ml soluble starch (2.5% w/v), 0.05ml 0.2mM EDTA, 0.05ml 20mM ADPG (or UDPG) containing 0.05μCi ADP-U^{14}C-glucose (or UDP-U^{14}C-glucose), and 0.05ml enzyme preparation. It was incubated for precisely 15 min at 30°C, and the reaction was stopped by the addition of 1ml chilled methanol-KCl solution (75% methanol, 1% KCl). Starch was precipitated by the addition of solid carrier starch, and pelleted by centrifugation at 10000g for 10 min. The pellet was washed in about 0.5ml buffer, re-centrifuged and suspended in 1ml buffer. This sample was then suspended in 2ml Beckman Ready-Solv MP scintillant (a gelling scintillant) and counted with a Beckman LS 100C liquid scintillation counter for 20 min.

Controls in which the enzyme preparation was replaced by distilled water were performed to determine how many of the counts were due to labelled ADPG or UDPG not removed in the washing and centrifuging steps. In order to determine how much starch was lost in these steps, the total starch content of digestions before and after

the washing and centrifuging steps was measured using the method described in Section 2.2.4 starting at the pronase digestion step.

To confirm that a glucose polymer was formed, a double-sized assay was performed which was incubated for 2h to get maximum incorporation of the ^{14}C-glucose. This was washed and centrifuged as before and the pellet was hydrolysed by boiling for 1h in 2ml 2N HCl. The resulting solution was freeze dried and the crystals taken up in 0.5ml glucose (2mg/ml). The product was chromatographed with either ^{14}C-glucose or a selection of monosaccharide standards by descending paper chromatography on Whatman No.1 chromatography paper in ethyl acetate / pyridine / water (8/2/1) for 48h. The chromatogram with the labelled standard was cut into strips and counted as described in Section 6.2.7. The one with the unlabelled standards was sprayed with a carbohydrate stain (3% para-anisidine in butanol / ethanol / water - 4/1/1 with a trace of stannous chloride), air dried and baked at 100°C for 3-5 min.

5.3 Results.

5.3.1 Glasshouse Experiment.

Photographs illustrating the difference in size of the ears and grains of cvs. Fenman and Broom are shown in Figs. 5.1 and 5.2.

Maximum caryopsis dry weight of both cultivars (Fig. 5.3a and b) was reached at 35 "days" after anthesis. The final dry weight per caryopsis was higher in Fenman than Broom. In Broom, fresh weight also peaked at 35 "days", but in Fenman fresh weight showed more of a plateau between 30 and 35 "days" before declining. Fenman also had the higher fresh weight. Water content (Fig. 5.3c) showed a plateau level

Fig. 5.1. Ears of Fenman (left) and Broom aged 25 days after anthesis.

(a)

(b)

Fig. 5.2. Caryopses of Fenman (left) and Broom (a) 25 days after anthesis and (b) 40 days after anthesis.

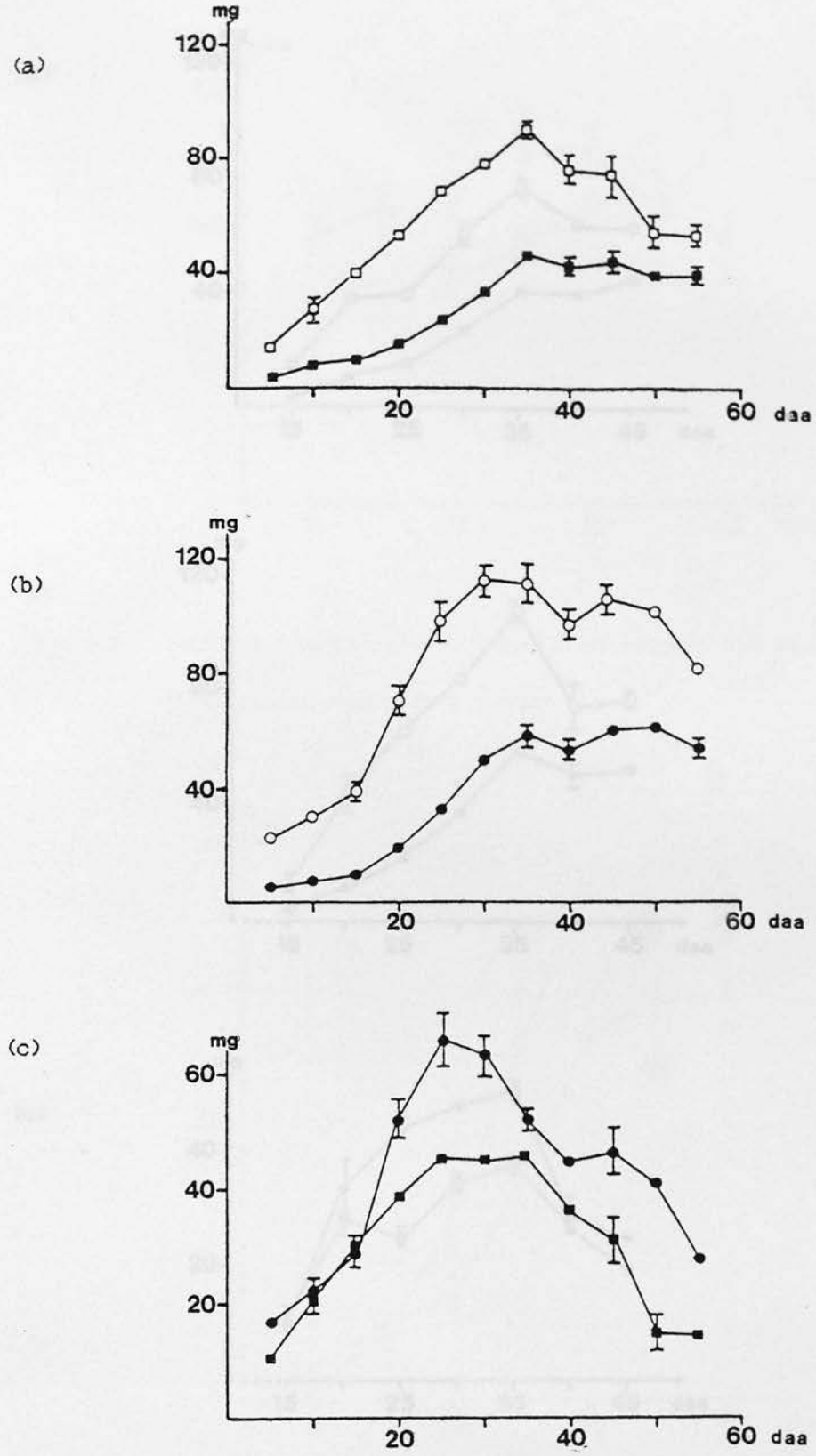


Fig. 5.3. Caryopsis fresh weight (□,○) and dry weight (■,●) of (a) Broom and (b) Fenman; and (c) water content of Fenman (●) and Broom (■) from glasshouse-grown plants.

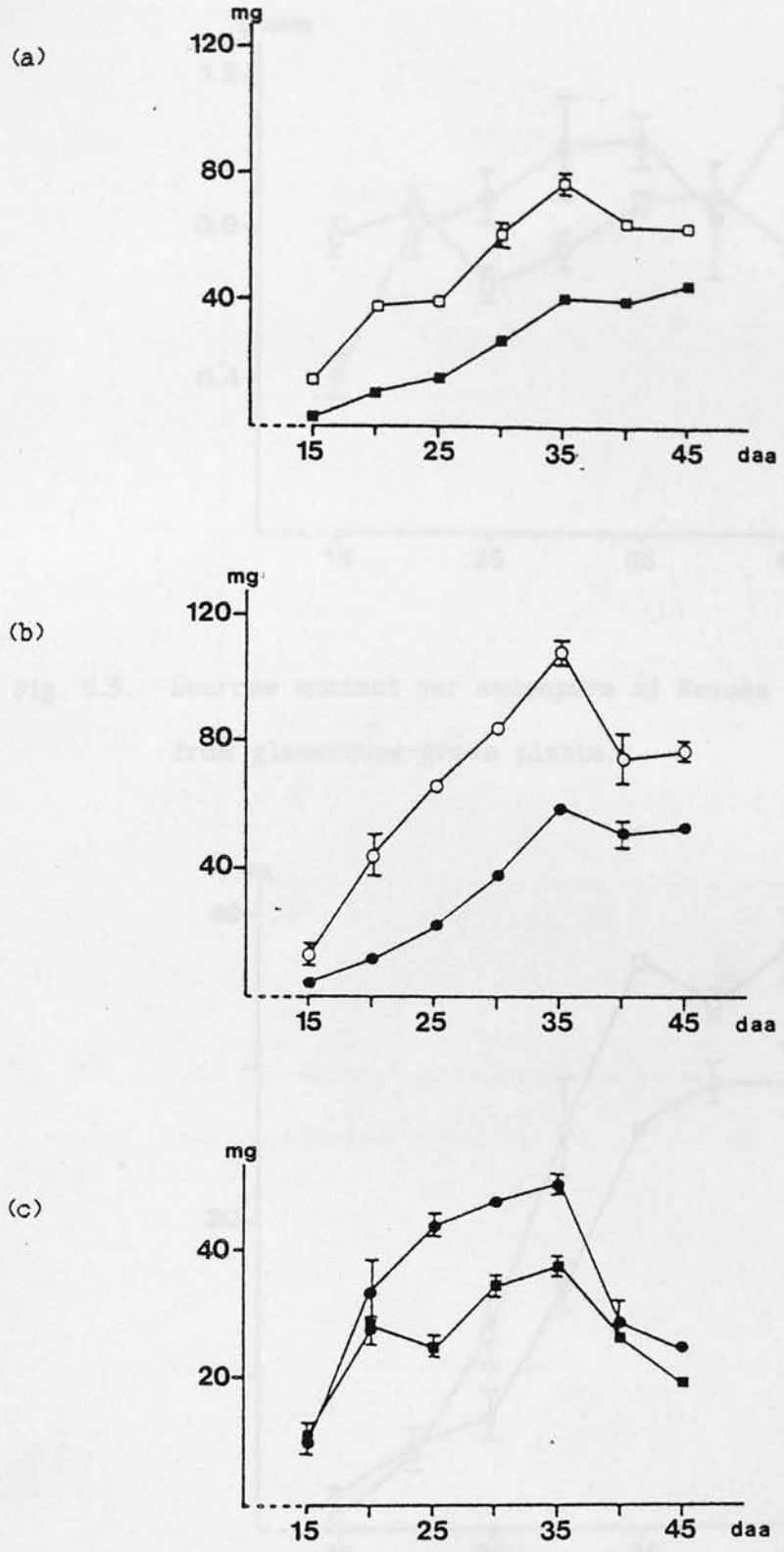


Fig. 5.4. Endosperm fresh weight (□,○) and dry weight (■,●) of (a) Broom and (b) Fenman; and (c) water content of Fenman (●) and Broom (■) from glasshouse-grown plants.

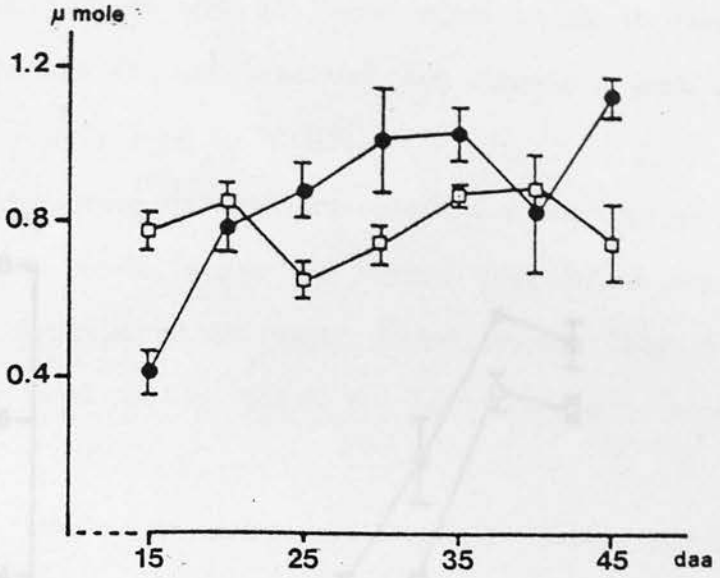


Fig. 5.5. Sucrose content per endosperm of Fenman (●) and Broom (□) from glasshouse-grown plants.

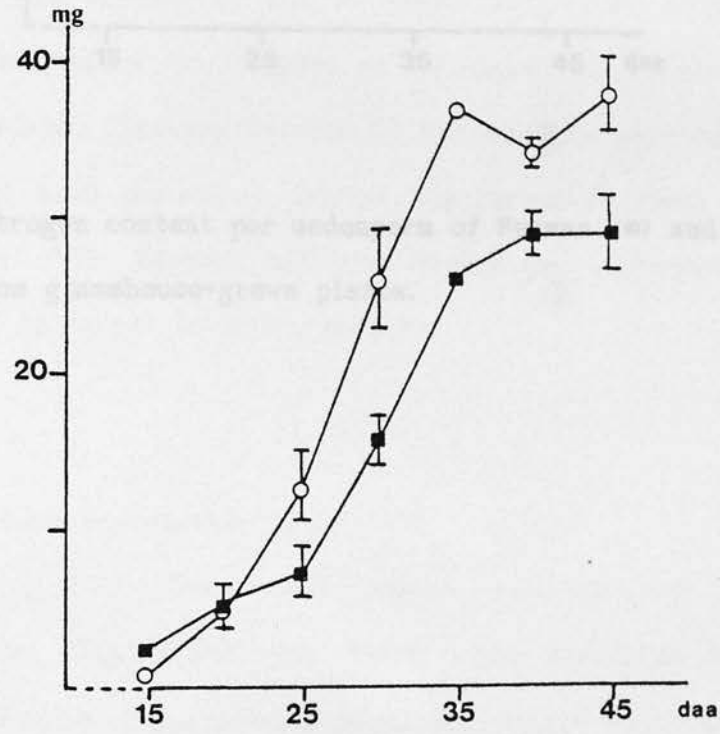


Fig. 5.6. Starch content per endosperm of Fenman (○) and Broom (■) from glasshouse-grown plants.

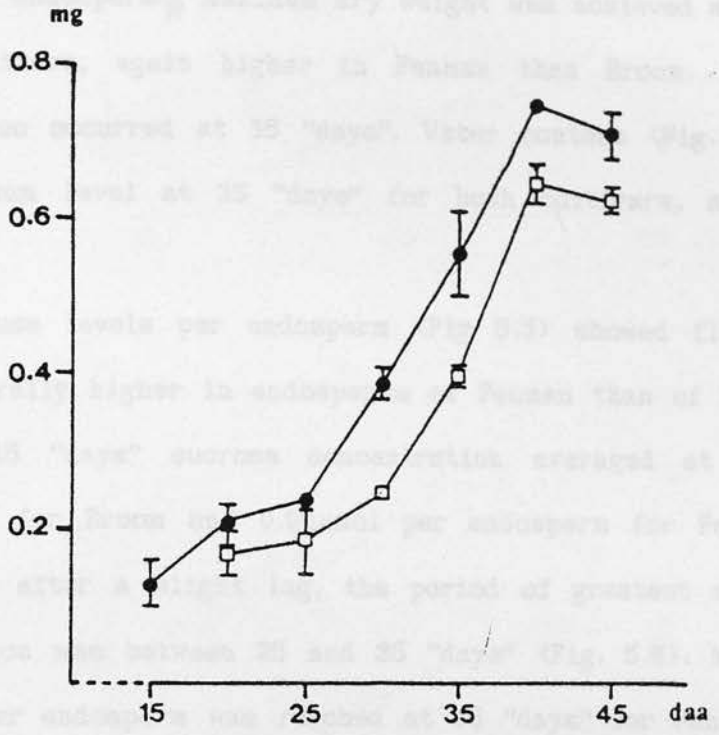


Fig. 5.7. Nitrogen content per endosperm of Fenman (●) and Broom (□) from glasshouse-grown plants.

for Broom between 25 and 35 "days" after which it declined. Water content of Fenman did not level off, but reached a peak at 25 "days" and declined slowly from 30 "days".

With endosperms, (Fig 5.4) maximum dry weight was achieved at 35 "days" in both cultivars, again higher in Fenman than Broom. Maximum fresh weight also occurred at 35 "days". Water content (Fig. 5.4c) reached its maximum level at 35 "days" for both cultivars, after which it dropped.

Sucrose levels per endosperm (Fig 5.5) showed fluctuations but were generally higher in endosperms of Fenman than of Broom. Between 20 and 45 "days" sucrose concentration averaged at 0.82μmol per endosperm for Broom and 0.96μmol per endosperm for Fenman. In both cultivars, after a slight lag, the period of greatest rate of starch accumulation was between 25 and 35 "days" (Fig. 5.6). Maximum starch content per endosperm was reached at 35 "days" for Fenman. In Broom, there was a slight increase between 35 and 40 "days". Nitrogen content per endosperm also showed an initial lag period followed by a rapid increase (Fig. 5.7). Highest nitrogen levels per endosperm were not reached until 40 "days" in either cultivar.

5.3.2 Growth Room Experiment.

In the 15/10°C temperature regime, maximum dry weight for cultivar Broom (Fig. 5.8a) was found from about 43 days after anthesis. In Fenman (Fig. 5.8b), a final dry weight was achieved at 41 days which was higher than that found with Broom. Corresponding with these, peak fresh weights were found at 43 days for Broom and 38 days for Fenman. Water content (Fig. 5.8c) maintained a fluctuating level between 20 and 38 days in Fenman before declining. In Broom, there

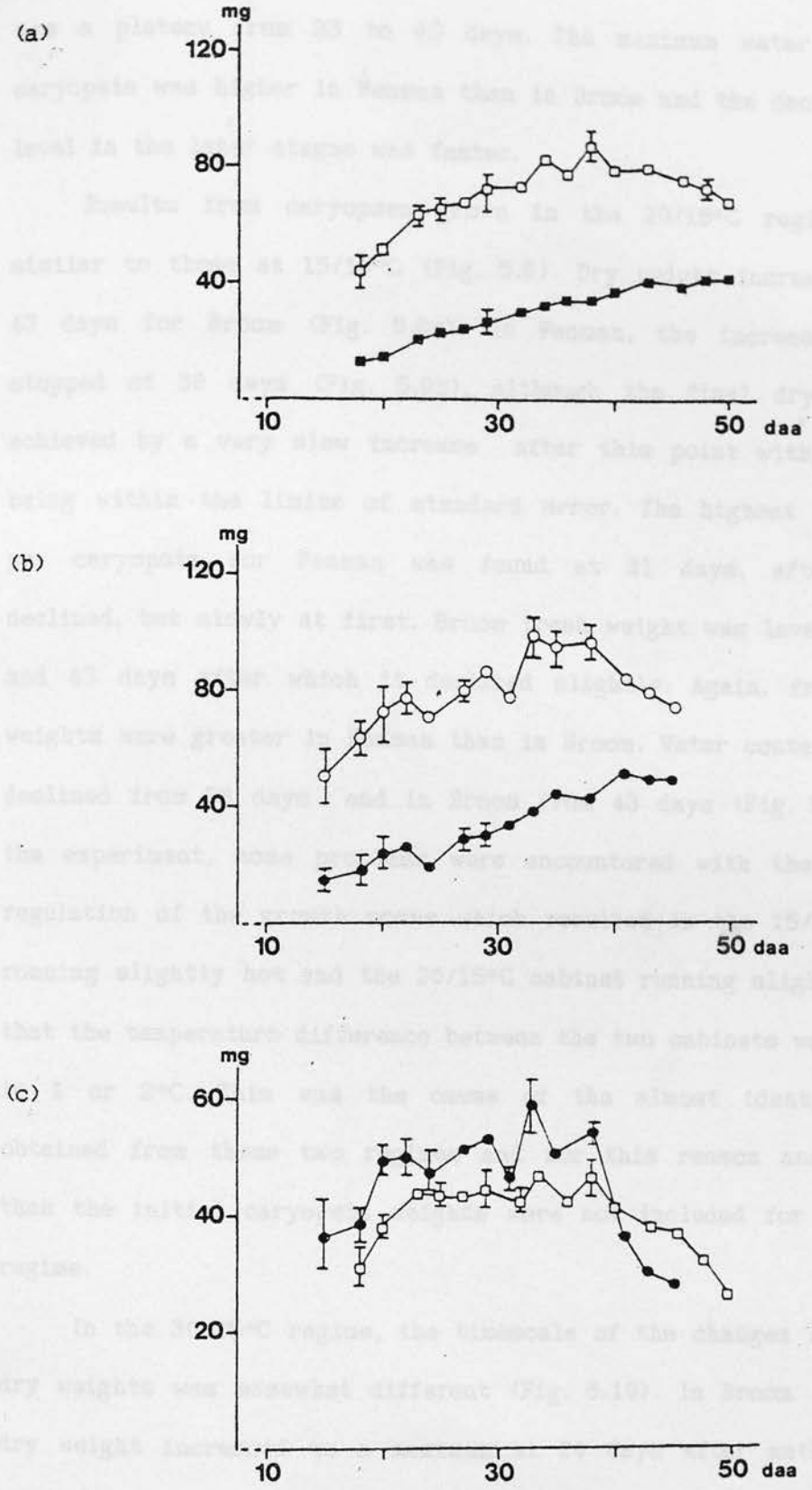


Fig. 5.8. Caryopsis fresh weight (□,○) and dry weight (■,●) of (a) Broom and (b) Fenman; and (c) water content of Fenman (●) and Broom (□) grown at 15/10°C.

was a plateau from 23 to 40 days. The maximum water content per caryopsis was higher in Fenman than in Broom and the decline in water level in the later stages was faster.

Results from caryopses grown in the 20/15°C regime are very similar to those at 15/10°C (Fig. 5.9). Dry weight increase ceased at 43 days for Broom (Fig. 5.9a). In Fenman, the increase apparently stopped at 38 days (Fig. 5.9b), although the final dry weight was achieved by a very slow increase after this point with the changes being within the limits of standard error. The highest fresh weight per caryopsis for Fenman was found at 31 days, after which it declined, but slowly at first. Broom fresh weight was level between 38 and 43 days after which it declined slightly. Again, fresh and dry weights were greater in Fenman than in Broom. Water content in Fenman declined from 38 days and in Broom from 43 days (Fig. 5.9c). During the experiment, some problems were encountered with the temperature regulation of the growth rooms which resulted in the 15/10°C cabinet running slightly hot and the 20/15°C cabinet running slightly cool, so that the temperature difference between the two cabinets was reduced to 1 or 2°C. This was the cause of the almost identical results obtained from these two regimes and for this reason analyses other than the initial caryopsis weights were not included for the 15/10°C regime.

In the 30/25°C regime, the timescale of the changes in fresh and dry weights was somewhat different (Fig. 5.10). In Broom (Fig. 5.10a), dry weight increased to a maximum at 24 days after anthesis. Fresh weight was also maximal at this point, after which it declined. In Fenman (Fig. 5.10b), these changes took place earlier, with dry weight increase levelling off at 21 days. Fresh weight fell from this point. Final dry weight was slightly lower in Fenman than in Broom. In

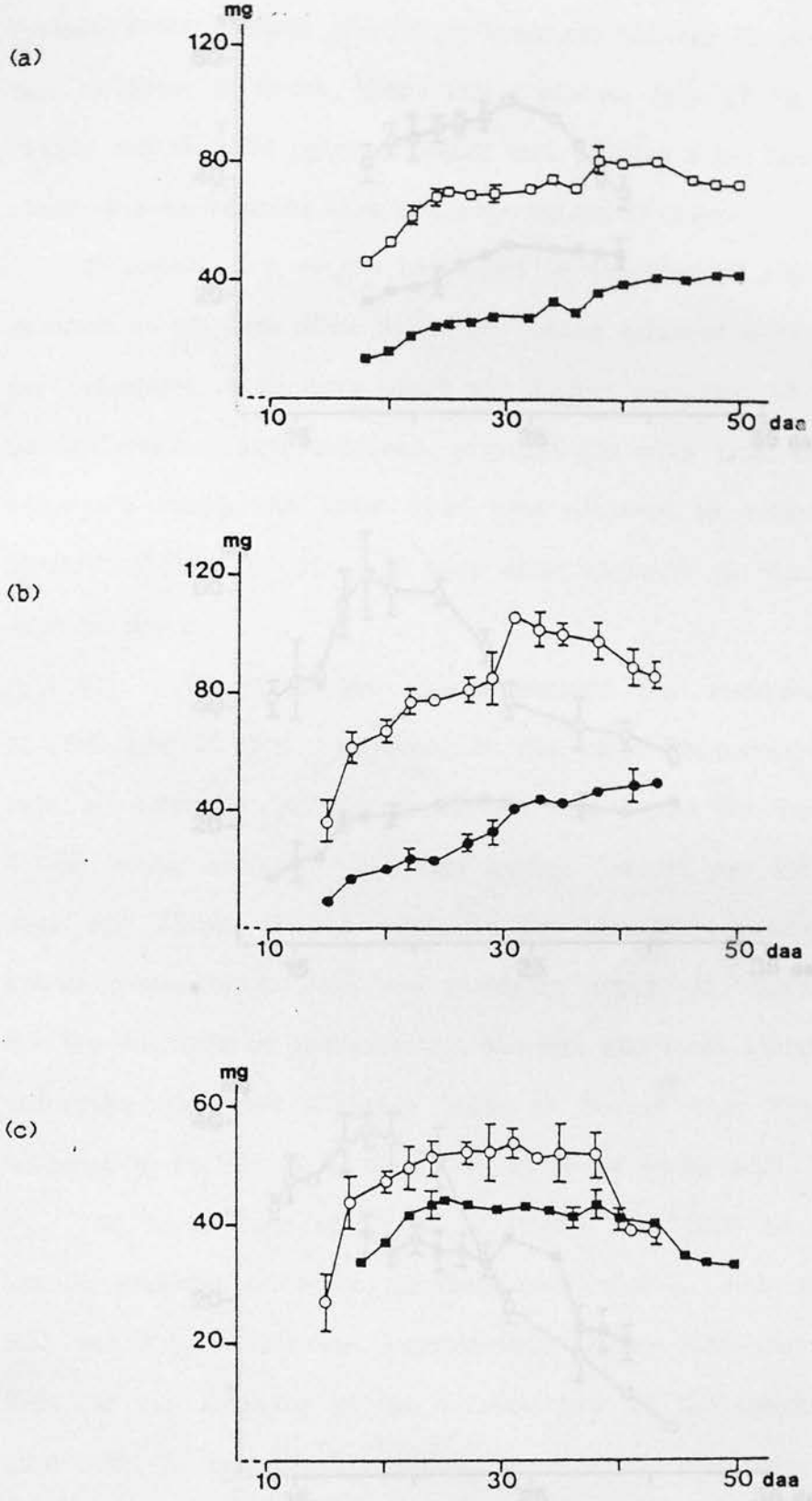


Fig. 5.9. Caryopsis fresh weight (□,○) and dry weight (■,●) of (a) Broom and (b) Fenman; and (c) water content of Fenman (○) and Broom (■) grown at 20/15°C.

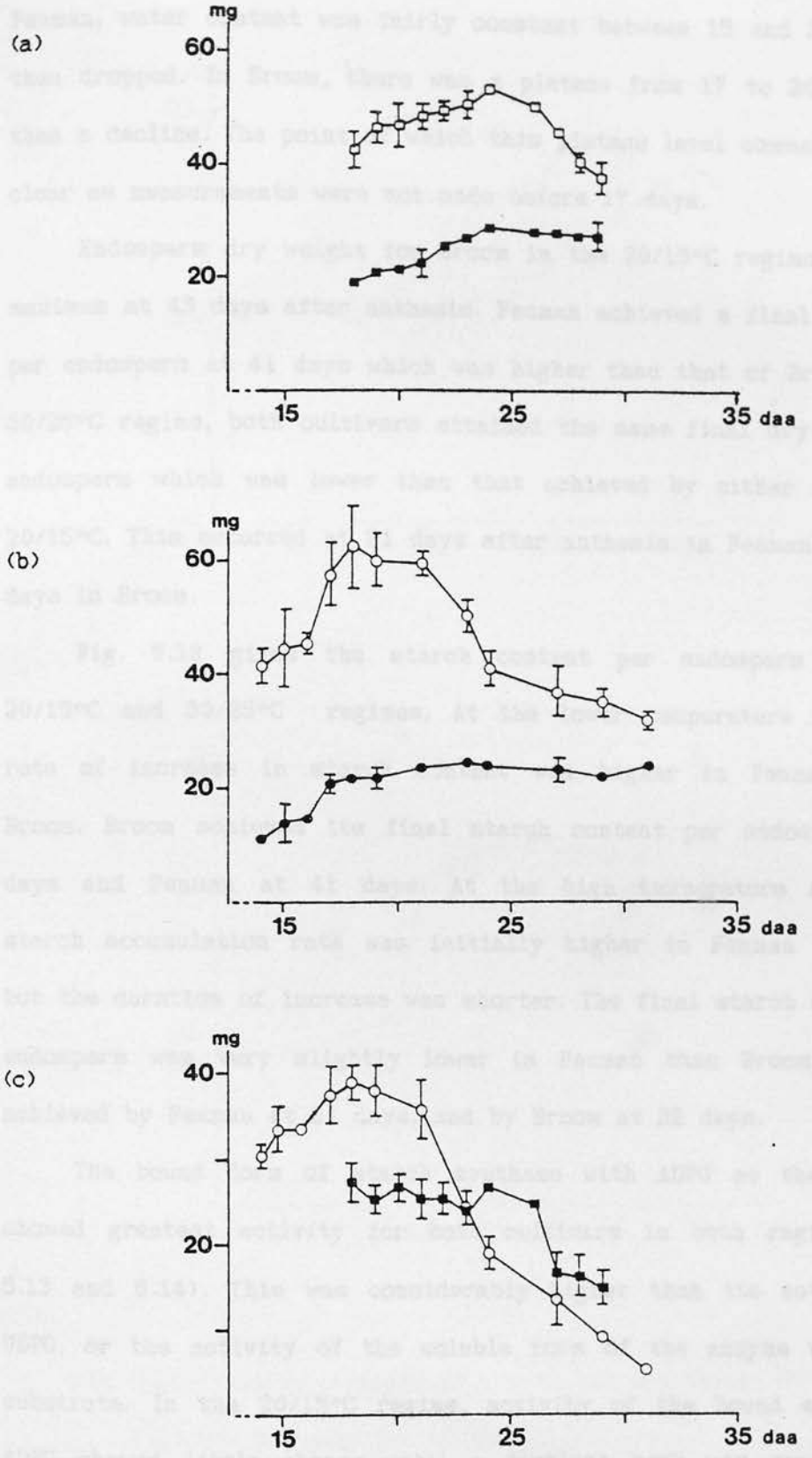


Fig. 5.10. Caryopsis fresh weight (□,○) and dry weight (■,●) of (a) Broom and (b) Fenman; and (c) water content of Fenman (○) and Broom (■) grown at 30/25°C.

Fenman, water content was fairly constant between 15 and 21 days and then dropped. In Broom, there was a plateau from 17 to 26 days and then a decline. The point at which this plateau level commences is not clear as measurements were not made before 17 days.

Endosperm dry weight for Broom in the 20/15°C regime, reached a maximum at 43 days after anthesis. Fenman achieved a final dry weight per endosperm at 41 days which was higher than that of Broom. In the 30/25°C regime, both cultivars attained the same final dry weight per endosperm which was lower than that achieved by either cultivar at 20/15°C. This occurred at 21 days after anthesis in Fenman, and at 22 days in Broom.

Fig. 5.12 gives the starch content per endosperm under the 20/15°C and 30/25°C regimes. At the lower temperature regime, the rate of increase in starch content was higher in Fenman than in Broom. Broom achieved its final starch content per endosperm at 43 days and Fenman at 41 days. At the high temperature regime, the starch accumulation rate was initially higher in Fenman than Broom but the duration of increase was shorter. The final starch content per endosperm was very slightly lower in Fenman than Broom. This was achieved by Fenman at 21 days, and by Broom at 22 days.

The bound form of starch synthase with ADPG as the substrate showed greatest activity for both cultivars in both regimes (Figs. 5.13 and 5.14). This was considerably higher than its activity with UDPG, or the activity of the soluble form of the enzyme with either substrate. In the 20/15°C regime, activity of the bound enzyme with ADPG showed little change until a distinct peak was reached at 43 days in both cultivars (Fig. 5.13a and 5.14a). Activity per endosperm was higher in Fenman than Broom. Levels of activity of the other three combinations of enzyme and substrate were all below 10pkatals per

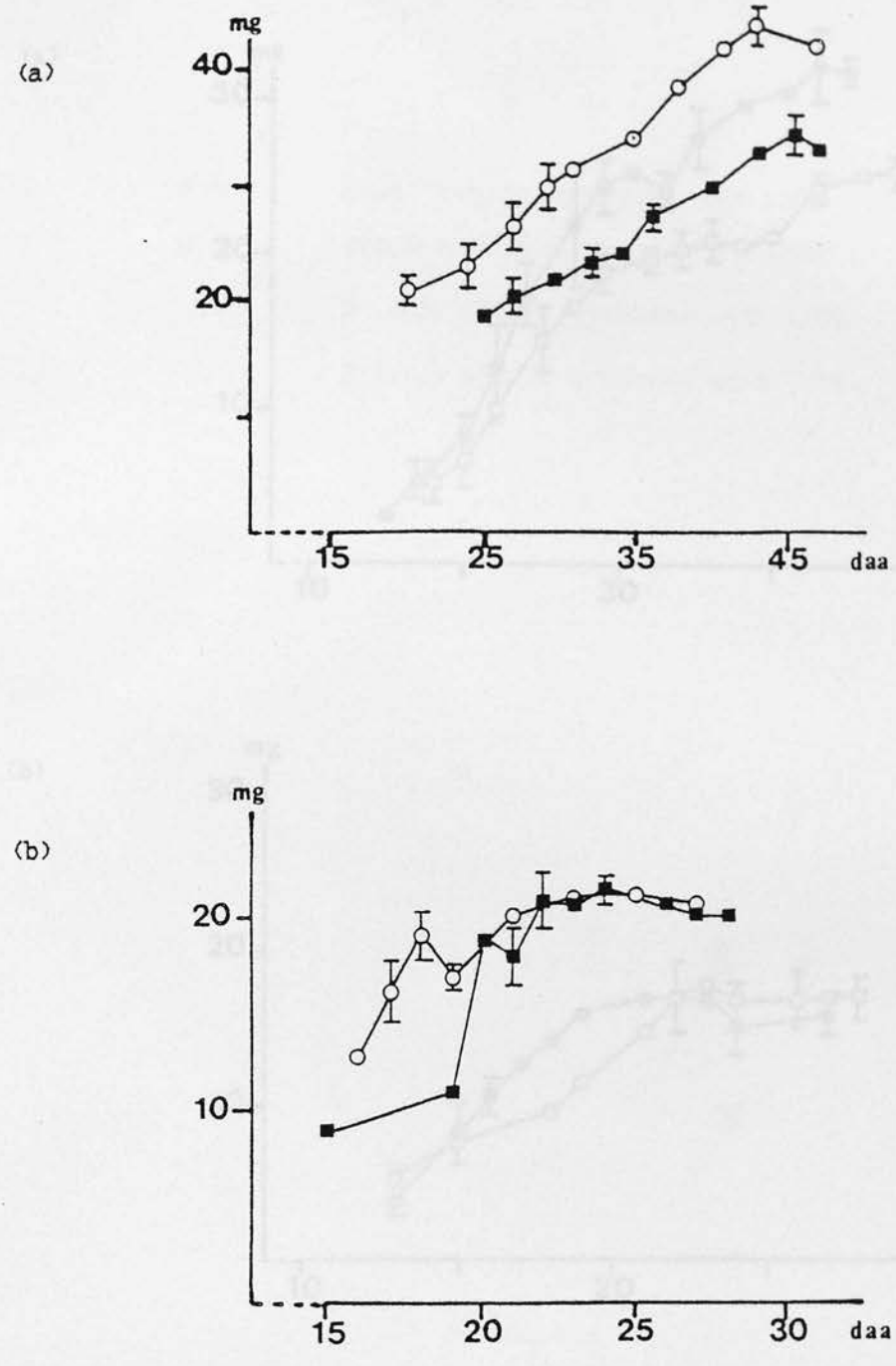


Fig. 5.11. Endosperm dry weight of Fenman (O) and Broom (■) grown at (a) 20/15°C and (b) 30/25°C.

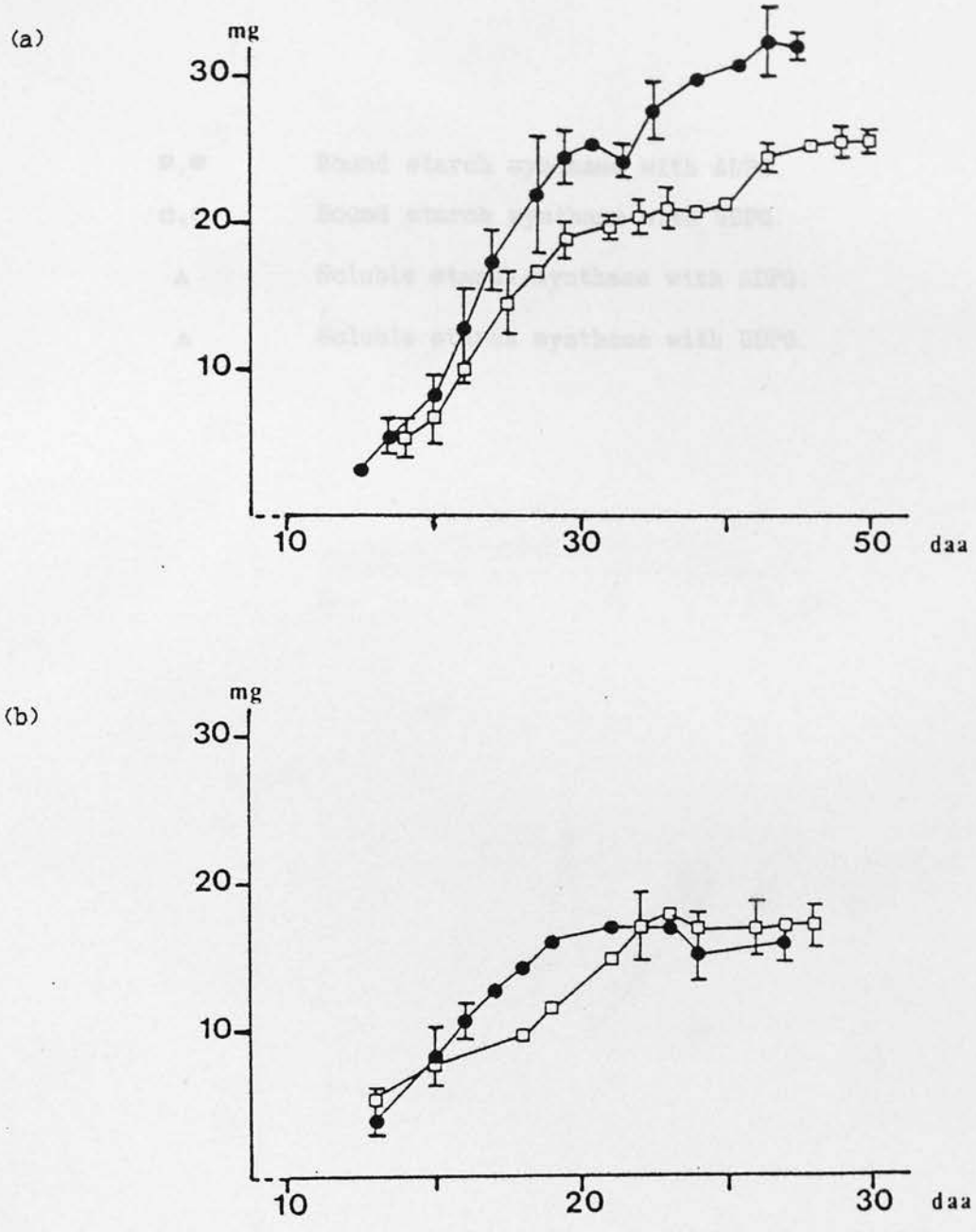


Fig. 5.12. Starch content per endosperm of Fenman (●) and Broom (□) grown at (a) 20/15°C and (b) 30/25°C.

Key to Figs. 5.13 and 5.14.

■,● Bound starch synthase with ADPG.

□,○ Bound starch synthase with UDPG.

▲ Soluble starch synthase with ADPG.

△ Soluble starch synthase with UDPG.

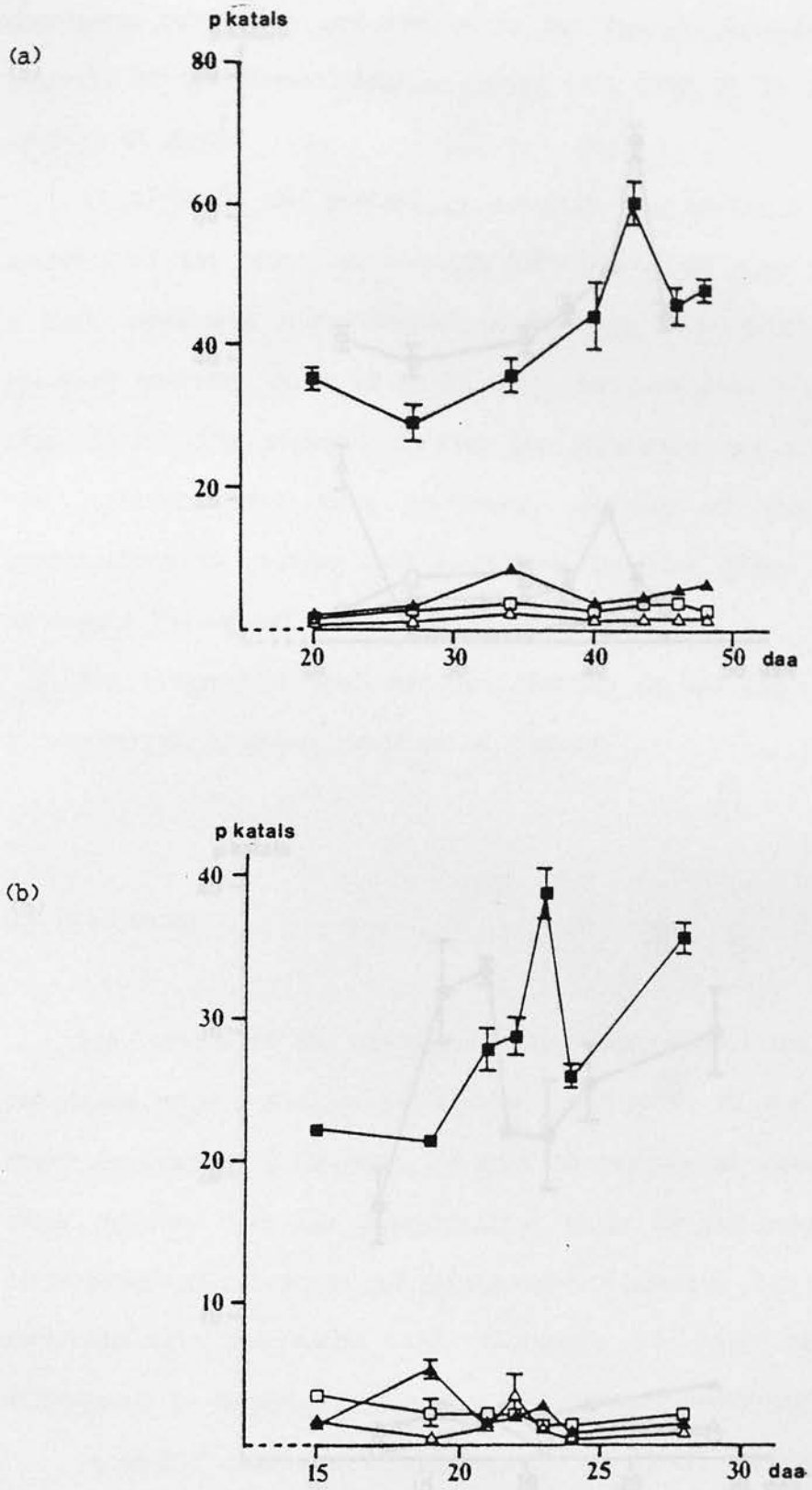


Fig. 5.13. Starch synthase activity per endosperm in Broom grown at (a) 20/15°C and (b) 30/25°C.

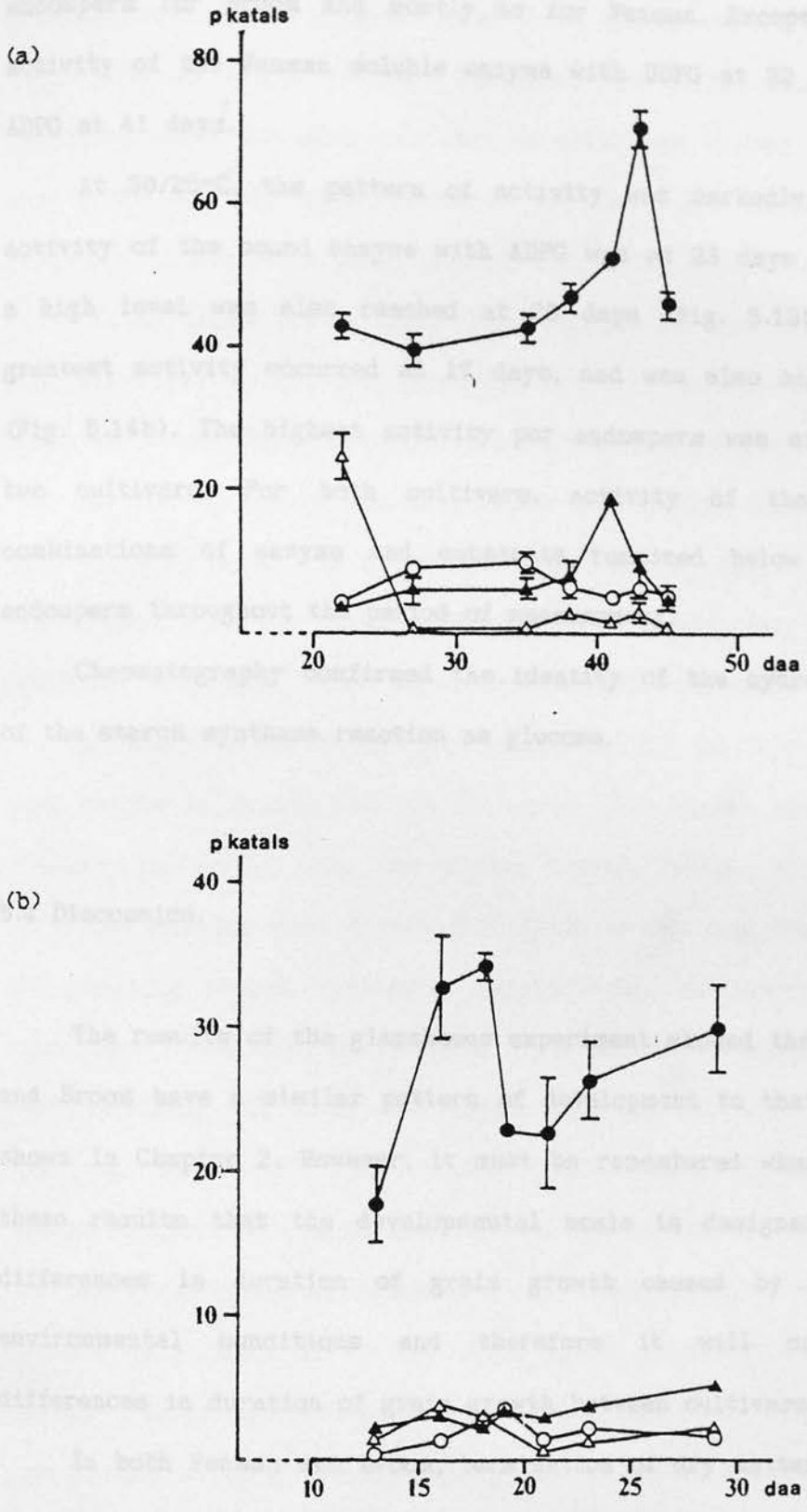


Fig. 5.14. Starch synthase activity per endosperm in Fenman grown at (a) 20/15°C and (b) 30/25°C.

endosperm for Broom and mostly so for Fenman. Exceptions were the activity of the Fenman soluble enzyme with UDPG at 22 days and with ADPG at 41 days.

At 30/25°C, the pattern of activity was markedly altered. Peak activity of the bound enzyme with ADPG was at 23 days for Broom and a high level was also reached at 28 days (Fig. 5.13b). In Fenman, greatest activity occurred at 17 days, and was also high at 29 days (Fig. 5.14b). The highest activity per endosperm was similar for the two cultivars. For both cultivars, activity of the other three combinations of enzyme and substrate remained below 5pkatals per endosperm throughout the period of measurement.

Chromatography confirmed the identity of the hydrolysed product of the starch synthase reaction as glucose.

5.4 Discussion.

The results of the glasshouse experiment showed that both Fenman and Broom have a similar pattern of development to that of cv. Sicco shown in Chapter 2. However, it must be remembered when interpreting these results that the developmental scale is designed to minimise differences in duration of grain growth caused by variations in environmental conditions and therefore it will not show any differences in duration of grain growth between cultivars.

In both Fenman and Broom, termination of dry matter accumulation was shown to occur at 35 "days" after anthesis, rather than at 40 "days" as for Sicco. This type of discrepancy was demonstrated by MacLeod (1986) working with similar developmental scales for barley cultivars, namely that different cultivars show slightly different

developmental patterns apart from those due to variations in duration and rate of grain filling. Therefore one would ideally require a separate scale for each cultivar. In this case it was not possible to carry out a field trial necessary for the large amounts of material required to establish developmental scales for these cultivars.

Given these limitations, these results do show that, as expected, the final dry weight per caryopsis was considerably higher in Fenman than in Broom. This was reflected in fresh weights too, and the water content per caryopsis was also higher for Fenman. Endosperm fresh and dry weights, water and starch contents were all higher in Fenman than in Broom, and for both cultivars dry weight and starch content levelled off at 35 "days" when fresh weight and water content started to decline. Starch content accounted for 69% of the final endosperm dry weight in Fenman and 73% in Broom. The higher sucrose content in Fenman correlates with the higher starch levels. As in Sicco, this does not decline when starch deposition ceases and does not appear to be limiting starch synthesis. Unlike Sicco, nitrogen content of the endosperm continues to increase after starch deposition has ceased, not levelling off until 40 "days". This agrees with the results of Barlow, Lee and Vesk (1974). Similarly, Cochrane (personal communication) has found that protein deposition in the aleurone layer of barley endosperms continues until slightly after the cessation of starch deposition. It is not clear which component of endosperm dry weight drops to accommodate the increase in nitrogen-containing compounds. There is a slight drop in the starch content of endosperms of Fenman but this is within the limits of error and there is no such drop in Broom. However, the increase in actual nitrogen, rather than any component it is assumed to represent, is very small compared to starch or dry weight levels of the endosperm and would not register a

difference on those scales. It is likely that deposition of nitrogen-containing compounds actually ceases very soon after termination of starch deposition but the intervals of measurement are sufficiently wide for it sometimes to appear to occur at the same time as termination of starch deposition and sometimes later.

In the growth room experiment, results were not adjusted to the developmental scale as the precise control of environmental conditions made this unnecessary. The closeness of the points of measurement in this experiment was intended to determine a precise order of events. However, the natural variation between grains, which at the "usual" measurement interval of 5 days would not have been noticed, led to overlapping of results at the closer intervals. This made it difficult to determine at what point differences occurred since they were obscured by the error bars. The results of the caryopsis weights are a good example of this: it is difficult to tell distinguish increase in dry weight from fluctuations resulting from differences between caryopses. The points mentioned in the results section are those at which it seems that an event has definitely occurred. It seems therefore that the increase in number of observations has not led to the hoped-for improvement in definition of slight but significant differences.

There were small differences between glasshouse-grown material and that from the lower temperature regimes in the growth rooms. The actual levels of caryopsis dry weight, endosperm dry weight and starch content were slightly lower than was found for the glasshouse material. This was possibly due to the artificial environment not being quite as suitable as the relatively natural one in some way, for example in light quality. Another possible cause is that the pot system used for this experiment differed from the one used in the

glasshouse. The individual pots used were considerably smaller than those used in the glasshouse and may have limited root growth which in turn could have limited growth of the rest of the plant.

As with glasshouse-grown material, dry weight increase in the caryopses of both cultivars ceases at around the time of maximum fresh weight and at the point where water loss commences. Since the results reported here are not adjusted to the developmental scale, it is also possible to comment on the rate and duration of grain-filling. At 15/10 °C and 20/15°C, the duration of dry matter deposition for Fenman was shorter than that for Broom. The final dry weight per caryopsis was also higher in Fenman than Broom and this was the result of a higher rate of dry matter accumulation during the filling period. Changes in caryopsis dry weight at 20/15°C were paralleled by changes in endosperm dry weight and endosperm starch content as in glasshouse-grown material. However, when starch is expressed as a percentage of the endosperm dry weight, figures of 74% for Fenman and 76% for Broom are obtained which are slightly higher than those obtained in the glasshouse. This implies that some factor contributing to dry weight is restricted more than starch deposition. It is not possible from these results to speculate on what this might be.

The 30/25°C regime was intended as a stress temperature and had a severe effect on development. The duration of the grain filling period was drastically curtailed and the final dry weight was also considerably lower than at the 20/15°C treatment. The effect of the stress temperature appears to have been greater on Fenman than on Broom as the final dry weight for Fenman was actually lower than that of Broom. Endosperm dry weight and starch content were also reduced compared to the 20/15°C regime, again with the greater reduction for Fenman. Expression of starch content as a percentage of endosperm dry

weight gives figures of 80% in Fenman and 83% in Broom. Thus starch content is relatively higher than at 20/15°C so some other component must have been affected to a greater extent and be contributing a relatively lower amount to dry weight in percentage terms. Again, there is insufficient information to speculate on what this component might be. It is unlikely to be nitrogen-containing compounds since other workers have found that nitrogen as a percentage of dry weight is also increased at elevated temperatures (Kolderup, 1975; Sofield, Wardlaw, Evans and Zee, 1977). Similar increases in starch content as a percentage of dry weight with raised temperatures can be calculated from the results of Spiertz (1977) for wheat grains and MacLeod (1986) for barley endosperms. Other workers have tended to look at the effect of temperature on either endosperm or grain dry weight or starch content, but not both, so it is not possible to determine whether or not there is an effect on percentage starch content of the endosperm.

Measurement of the activity of starch synthase demonstrated that most of its activity in the endosperm is that of the bound form with ADPG as the substrate. This is consistent with the results of Cardini and Frydman (1966) who found greater activity with ADPG than UDPG for starch synthase from a variety of storage tissues including wheat grains. Activity of the bound form with UDPG and of the soluble form with either substrate was very small by comparison. Baxter and Duffus (1973b) found activity of the soluble form from barley endosperms at 20 days after anthesis was much greater with UDPG than with ADPG and also greater than the activity of the bound form with UDPG. This agrees with the data reported here for Fenman at 22 days in the 20/15°C regime. It is possible that the soluble form is more important in the earlier stages of starch deposition than in the later stages. It

is not clear whether that is the case from the results presented here as measurements in the early stages were generally omitted in favour of the termination phase. However, Baxter (1972) found that the UDPG-linked soluble enzyme was predominant in barley endosperms in the first 10 days after anthesis. The small peak in activity of the Fenman soluble enzyme with ADPG was probably due to contamination with the bound enzyme.

The peak of activity per endosperm of the bound enzyme with ADPG came at about the same point as starch deposition stopped in both cultivars. This peak and the maintenance of activity after starch deposition had ceased at 20/15°C and at 30/25°C showed that it was not lack of starch synthase activity that caused the termination of starch deposition. A similar pattern of continued starch synthase activity after the termination of starch accumulation has been found in wheat grains by Mengel and Judel (1981) and Kumar and Singh (1984). The implications of this are considered further in the next chapter. The differences in activity between the two cultivars might be due to either a higher specific activity or a larger amount of the enzyme protein present in endosperms of Fenman.

Activity per endosperm of the bound starch synthase with ADPG was lower at 30/25°C than at 20/15°C in both cultivars with reduction in peak activity being greater for Fenman than Broom. This is consistent with the greater reduction in starch content occurring in Fenman compared to Broom. The lower activity per endosperm could be the result of a reduced specific activity or a lower amount of the enzyme protein or both at the higher temperature regime. Reduced starch synthase activity at elevated temperatures correlates with lower activity of UDP-sucrose synthase found in barley endosperms (MacLeod, 1986) and of several enzymes of starch synthesis including

starch synthase from potatoes (Mangat and Badenhuizen, 1971) found at temperatures of 30°C. The results from potatoes showed a reduction in specific activity of starch synthase, but it is unclear whether or not protein levels were also affected.

In summary, it has been shown that the higher final dry weight in Fenman is due, at least in part, to the greater amounts of starch deposited in the endosperm compared to Broom. This is achieved by a higher rate of starch deposition, with the duration of the deposition phase being slightly shorter in Fenman than Broom. Activity of the bound form of starch synthase appears to be important in determining the rate at which starch synthesis occurs. This is demonstrated by the reduction in starch content when starch synthase activity is reduced by elevated temperature. The greater reduction in starch synthase activity of Fenman resulted in a higher reduction in endosperm starch content compared to Broom. Also, the rates of starch deposition which can be calculated to be possible from the observed starch synthase activities are very similar to those recorded. The maintenance of starch synthase activity after starch deposition and dry matter accumulation ceases suggests that reduction in starch synthase activity is not a cause of termination of grain growth. However, if it were inhibited *in vivo*, it could be a very important factor affecting termination. This is considered further in the next chapter.

6. MANIPULATION OF STARCH SYNTHESIS.

6.1 Introduction.

The work reported in Chapter 5 showed that starch synthase remains active after starch deposition has ceased. There are at least three possible reasons for this. These are (1) that the substrate ADPG is unavailable to the enzyme; (2) that the enzyme is inhibited *in vivo*; or (3) that starch degradation is proceeding at the same rate as starch synthesis. The last of these suggestions seems unlikely since the results presented in Chapter 4 suggest that there is probably a general reduction in metabolic activity after the onset of water loss, which would not correlate with an increase in the activity of starch degrading enzymes. Results of studies on activity of amylases and phosphorylase during grain development show that for the most part their peak of activity is rather earlier in development, and there is not an increase in activity after the termination of dry matter deposition (Meredith and Jenkins, 1973; Duffus and Rosie, 1973b). In addition, scanning electron microscopy of starch granules from developing cereal grains does not show the pitting charcteristic of α-amylase activity (Duffus, personal communication).

In order to determine whether or not the substrate is limiting, it is necessary to measure levels of ADPG inside the amyloplast. This has been prevented by the absence of a method for the preparation of intact amyloplasts in amounts sufficient for analysis. Measurements of the level of ADPG in endosperms of cv. Sicco show no apparent decline for at least 5 days after the termination of dry matter accumulation (Riffkin, personal communication). Similarly, the results of Jenner

(1968b) show little change in ADPG level when starch accumulation terminates, but there is no way of telling where in the endosperm the ADPG is located. Recently, methods have been published for the isolation of intact amyloplasts from various sources (Gaynor and Galston, 1983; MacDonald and ap Rees, 1983: Sack, Priestly and Leopold, 1983; Echeverria, Boyer, Lui and Shannon, 1985). However, none of these reports have included convincing evidence that a sufficient percentage of the amyloplasts are intact, surrounded by a double membrane and free from cytoplasmic contaminants. It seems likely that accurate determination of metabolite levels in these organelles will require a great deal of further work.

The hypothesis to be tested in this section is that the cessation of starch synthesis is the result of *in vivo* inhibition. The concept of product inhibition is well known but it is highly improbable that it operates here since starch is a substrate of the reaction as well as a product. Studies on the effects of certain substances on starch synthase activity have tended to concentrate on those which cause activation. The unprimed activity of the soluble fraction from maize kernels has been shown to be stimulated by the presence of 0.5M citrate (Boyer and Preiss, 1979; Pollock and Preiss, 1980). One of the solubilized granule-bound fractions from maize kernels also shows considerable unprimed, citrate-stimulated activity (MacDonald and Preiss, 1983). When the enzyme is associated with a starch granule, several reports have indicated an increased activity in the presence of univalent cations (Murata and Akazawa, 1968; Nitsos and Evans, 1969; Hawker, Marschner and Downton, 1974; Rijven and Gifford, 1983). Potassium ions have been found to be most effective, and are an absolute requirement in the case of sweetcorn starch synthase (Nitsos and Evans, 1969). In leaves of several plants, it has been found that

both the bound and soluble enzymes are activated by sodium and potassium ions (Hawker, Marschner and Downton, 1974).

In the experiments described here, the effects on endosperm starch synthase activity of selected substances were investigated. These were potassium, phosphate, abscisic acid and polyethylene glycol. Potassium was used to demonstrate the activation effect in order to test whether or not starch synthase from wheat endosperm is sensitive to external factors in a similar manner to starch synthases from other plant sources. Phosphate has been shown to cause inhibition of ADPG pyrophosphorylase activity in wheat endosperms (Riffkin, personal communication) and other plant tissues (Ghosh and Preiss, 1966; Sanwal, Greenberg, Hardie, Cameron and Preiss, 1968), and it is possible that it may also affect starch synthase. Abscisic acid levels reach a peak at the point when starch deposition ceases and water loss starts (Golbach and Michael, 1976; Radley, 1976b; King, 1976), but its target has not been identified. It is possible that starch synthase activity could be affected either directly or indirectly. Polyethylene glycol was included in an attempt to simulate a low water environment since it is possible that it is simply lack of water which inhibits starch synthase activity *in vivo*. The effects of these substances were studied in whole ears using detached ear culture looking at their effects on starch deposition, and in the starch synthase assay to see if they had any direct effect on activity.

ADPG pyrophosphorylase activity was also measured in endosperms from the detached ear culture experiments. This was to determine whether or not the two enzymes were affected differently by the test substances and if changes in the activity of ADPG pyrophosphorylase could be affecting starch synthase activity by altering the ADPG supply.

6.2 Materials and Methods.

6.2.1 Plant Material.

Wheat plants, *Triticum aestivum* cv. Fenman and cv. Broom were grown in the glasshouse as described in Section 2.2.1. Stage of development was assessed according to the developmental scale for Sicco, omitting the size data.

6.2.2 Detached Ear Culture

The liquid culture system of Donovan and Lee (1977) was used. Ears of wheat were cut at the flag leaf node, the flag leaf removed, and the stem surface sterilized with a 10% sodium hypochlorite solution. The stem was then recut under sterile distilled water to a length of 15cm from the base of the ear.

Each ear was inserted loosely through a sterile cotton plug into a sterile glass vial containing 25ml of culture medium. The culture medium used was that described in Section 3.2.2, but with 0.12M sucrose (4%), the level which has been found to be optimal for detached ear culture (Campbell, Lee and O'Brien, 1981; Barlow, Donovan and Lee, 1983). Test substances being studied were incorporated into the medium during preparation at the following concentrations: 4μM abscisic acid (ABA), 20mM K_2SO_4, 10mM H_3PO_4, and 200g/l polyethylene glycol (PEG) 8000 (deionized on a column of ion-exchange resin Amberlite MB1). Culture vessels were maintained at 4°C in a shallow water bath to reduce any contamination of the media. Ears were transferred to fresh sterile media and vials every 4-6 days, with the stem being recut under sterile distilled water. All transfers were

carried out in a laminar flow cabinet to reduce microbial contamination.

Ears were maintained at the appropriate temperature in a Fisons 600H controlled environment cabinet, on an 18h day length and with a relative humidity of 60-65%. Illumination was 280μE m^{-2} s^{-1} at ear height. The culture period was 10 days, after which the ears were sampled.

6.2.3 Fresh and Dry Weights.

Endosperm fresh and dry weights were measured by the method described in Section 2.2.2.

6.2.4 Starch Determination

Starch was determined according to the method described in Section 2.2.4.

6.2.5 Assay for Starch Synthase Activity.

Bound starch synthase was extracted by the method in Section 5.2.4 and activity assayed by the method described in Section 5.2.5.

Kinetic experiments were performed using bulk extractions of 25 day endosperms of Fenman and Broom from the glasshouse so that aliquots of the same preparation were used for all measurements of each cultivar. ADPG at 0.25, 0.5, 1, 2 and 4mM was used. Test substances were used at the following concentrations: ABA at 0, 2, 4 and 10μM; K_2SO_4 at 0, 5, 10 and 20mM (= 0, 10, 20 and 40mM K^+); H_3PO_4

at 0, 5, 10 and 20mM; and PEG 8000 at 0, 20, 100 and 200g/l. Assays were performed in duplicate.

Temperature and pH profiles of starch synthase activity were also performed. Activity at temperatures in the range 15-40°C was assayed. Activity over the pH range pH6.5-9.0 was measured using the following buffers: pH6.5-7.5 MOPS, pH8.0-8.5 glycylglycine and pH9,0 bicine.

6.2.6 Extraction of ADPG Pyrophosphorylase.

Endosperms (10-12) were homogenised on ice in about 2ml ice-cold 50mM MOPS buffer pH 7.5 containing 5mM dithiothreitol, 0.2mM ATP, 10mM $MgCl_2$ and 2% w/v glycerol. The homogenate was centrifuged at 10000g for 15 min at 0-4°C. The supernatant was saved on ice, and the pellet re-extracted in 0.5ml buffer and re-centrifuged. The combined supernatants formed the ADPG pyrophosphorylase preparation.

6.2.7 Assay for ADPG Pyrophosphorylase Activity.

ADPG-pyrophosphorylase (ATP: α-D-glucose-1-phosphate adenylyl-transferase, E.C. 2.7.7.27) was assayed by the method of Riffkin (personal communication) based on the methods of Shen and Preiss (1964) and Ghosh and Preiss (1966). The reaction mixture comprised 20µl 100mM HEPES buffer pH8.0, 10µl 50mM $MgCl_2$, 10µl 12mM ATP, 10µl 6.8mM glucose-1-phosphate containing 0.74µCi μmol^{-1} ^{14}C-glucose-1-phosphate, 5µl (0.625U) inorganic pyrophosphatase (Sigma Chemical Co. Ltd., 500U prepared in 4mls 50mM HEPES pH8.0) and 45µl enzyme preparation. The mixture was incubated at 25°C for precisely 10 min and the reaction terminated by boiling for 45s. When cool, 0.5U

alkaline phosphatase (Sigma Chemical Co. Ltd., pre-dialysed against 50mM HEPES pH8.0 for 1½-2h at 2°C) was added and the whole was incubated for a further 1½h at 25°C. An aliquot of the digest (60-100µl) was loaded onto a DEAE-cellulose paper strip (1.5x8cm), dried, washed three times in double-distilled water, and re-dried. The paper strip was then placed in a scintillation vial with 2ml scintillant (9g PBD and 0.5g POPOP per litre of toluene) and counted with a Beckman LS 100C liquid scintillation counter for 20 min. Each sample (of three replicates) was assayed in duplicate.

For each sample, a control without ATP was performed to indicate how many of the counts were due to glucose-1-phosphate which was not cleaved by the alkaline phosphatase and remained bound to the DEAE-cellulose paper. A control to determine how much ADPG was lost in the washing steps was achieved by including a known amount of ^{14}C-ADPG in the reaction mixture, omitting ^{14}C-glucose-1-phosphate and the enzyme preparation, and starting at the point of alkaline phosphatase digestion.

In order to confirm that the product of the reaction was ADPG, a double-sized assay was performed and loaded onto a double-sized DEAE-cellulose strip. This was taken through the washing and drying stages as usual, then the product was eluted from the paper with pyridine / acetic acid (5% pyridine solution, pH5 with acetic acid) and freeze dried. The crystals were taken up in 0.5ml ADPG (2mg/ml) and chromatographed by descending paper chromatography on Whatman No.1 chromatography paper in 71% aqueous ethanol containing 0.2M ammonium acetate and 0.003% EDTA at pH7.0 for 48h. ^{14}C-labelled standards of ADPG, UDPG and glucose-1-phosphate were co-chromatographed. After drying, the chromatogram was cut into strips and counted as previously described. To identify the sugar as glucose, a portion of

the eluted product was hydrolysed by boiling with 0.1M trifluoroacetic acid for 30 minutes. The resulting solution was freeze dried and the crystals taken up in 0.5ml glucose (2mg/ml). The product was chromatographed as described above, but the solvent was ethyl acetate / pyridine / water (8/2/1). Either ^{14}C-glucose or a range of unlabelled monosaccharide standards were used. The chromatogram with the labelled standard was cut into strips and counted. That with unlabelled standards was sprayed with a carbohydrate stain (3% w/v para-anisidine in butanol / ethanol / water - 4/1/1 with a trace of stannous chloride), air-dried and baked in an oven at 100°C for 3-5 min.

6.3 Results.

The system of detached ear culture used is illustrated in the photograph in Fig. 6.1. Due to limited space in the controlled environment cabinet, two separate culture experiments were performed. In each, a set of control ears was cultured on the ordinary medium while two other sets were used for culturing with two of the compounds being tested. In the first experiment these were potassium and ABA and in the second, phosphate and PEG.

In the first experiment, there was an increase in both fresh and dry weight of Fenman and Broom endosperms over the culture period shown by the higher weights from ears cultured on the ordinary medium compared to those at day 0 (Fig. 6.2). Dry weights of endosperms from the potassium and ABA treated ears were slightly lower compared to the untreated ears in Fenman, but were very similar in Broom. Starch content of the endosperm (Fig. 6.3) increased over the 10 day culture

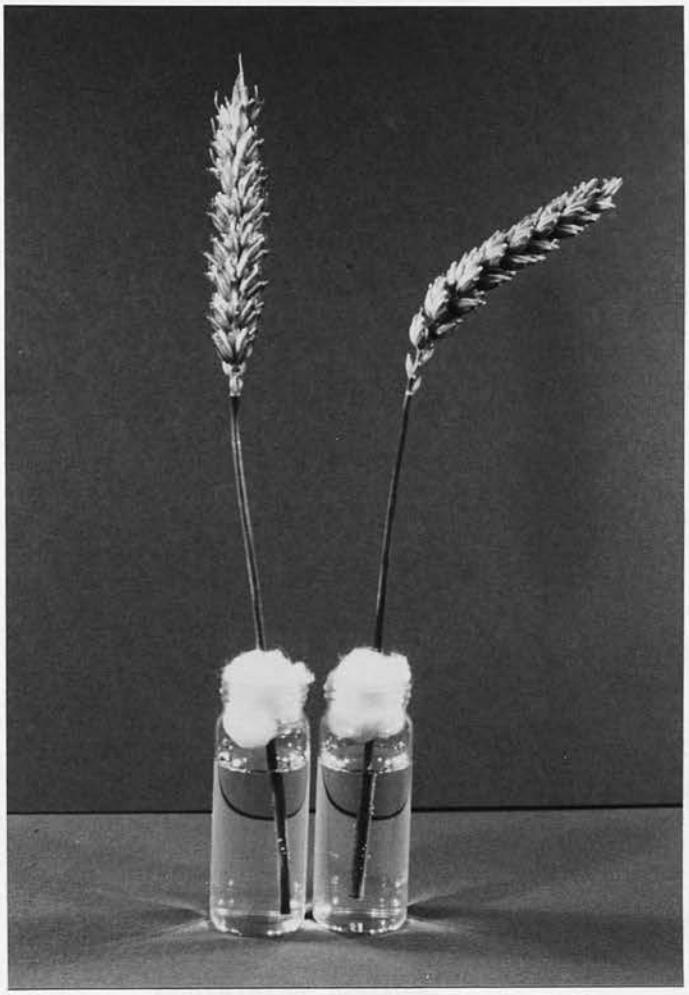


Fig. 6.1. Detached ear culture system with ear of Fenman (left) and Broom.

Key to Figs. 6.2 to 6.11.

Results of Detached Ear Culture Experiments.

Day 0 - Results from samples taken at the beginning of the culture period.

Day 10 - Results from samples taken after 10 days in culture.

Treatments:

O - Untreated control ears cultured on the ordinary medium

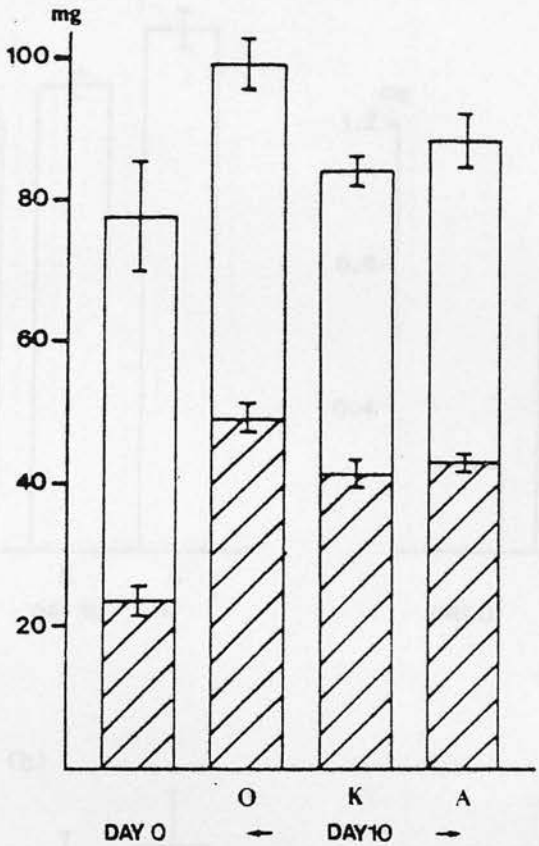
K - Ears cultured on potassium-containing medium.

A - Ears cultured on abscisic acid-containing medium.

Pi - Ears cultured on phosphate-containing medium.

PEG - Ears cultured on polyethylene glycol-containing medium.

(a)



(b)

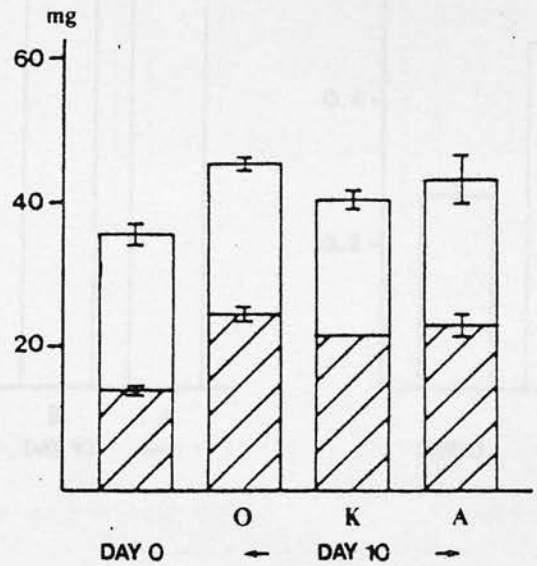
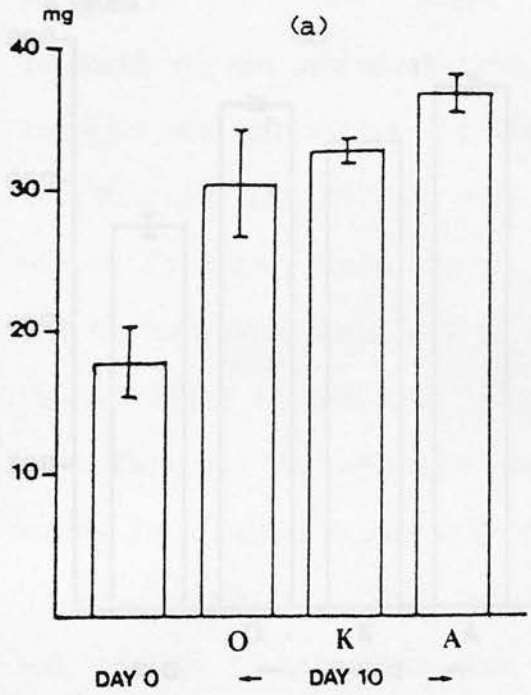


Fig. 6.2. Endosperm fresh weights (whole bars) and dry weights (shaded regions) of (a) Fenman and (b) Broom in the first detached ear culture experiment.



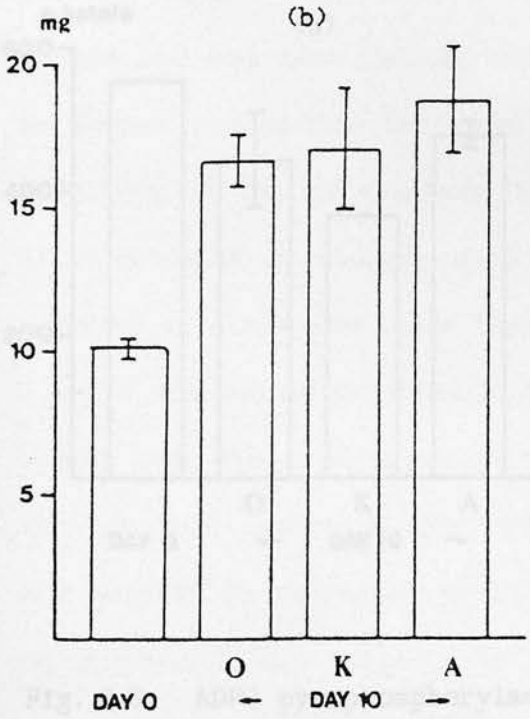
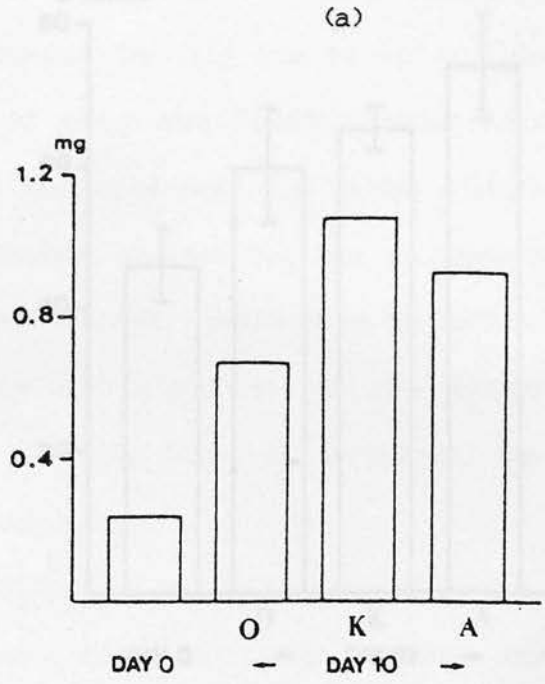


Fig. 6.3. Starch content per endosperm of (a) Fenman and (b) Broom in the first ear culture experiment.



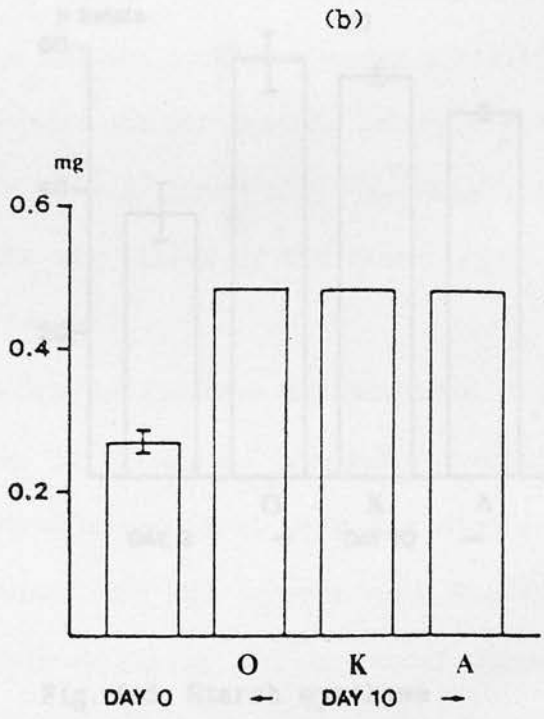
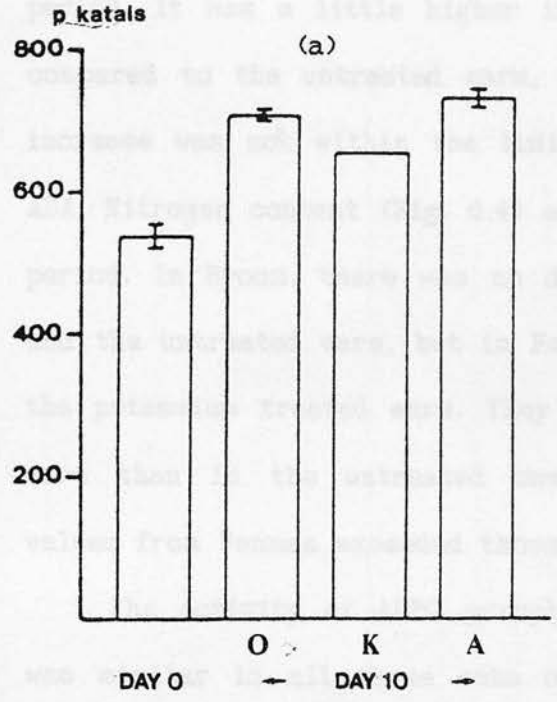


Fig. 6.4. Nitrogen content per endosperm of (a) Fenman and (b) Broom in the first ear culture experiment.



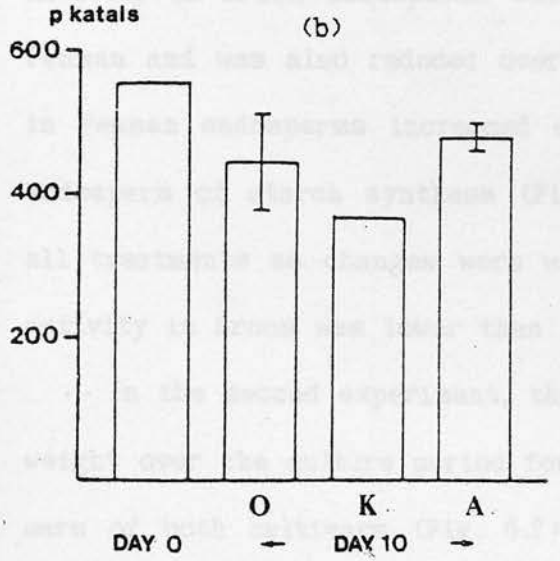
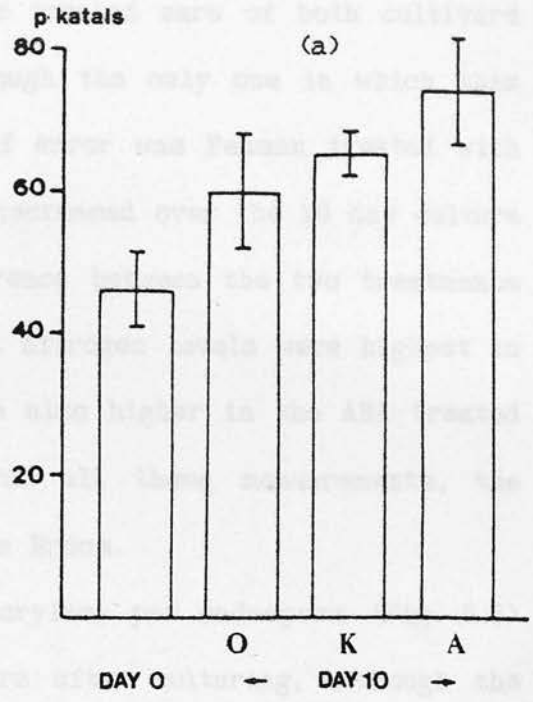


Fig. 6.5. ADPG pyrophosphorylase activity per endosperm of (a) Fenman and (b) Broom in the first ear culture experiment.



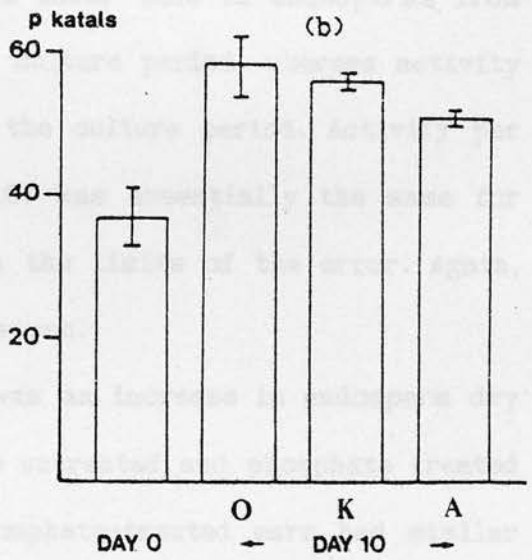


Fig. 6.6. Starch synthase activity per endosperm of (a) Fenman and (b) Broom in the first ear culture experiment.

period. It was a little higher in the treated ears of both cultivars compared to the untreated ears, although the only one in which this increase was not within the limits of error was Fenman treated with ABA. Nitrogen content (Fig. 6.4) also increased over the 10 day culture period. In Broom, there was no difference between the two treatments and the untreated ears, but in Fenman nitrogen levels were highest in the potassium treated ears. They were also higher in the ABA treated ears than in the untreated ones. For all these measurements, the values from Fenman exceeded those from Broom.

The activity of ADPG pyrophosphorylase per endosperm (Fig. 6.5) was similar in all three sets of ears after culturing, although the potassium treated ears showed slightly lower activity. However, activity in Broom endosperms was much lower than in endosperms from Fenman and was also reduced over the culture period, whereas activity in Fenman endosperms increased over the culture period. Activity per endosperm of starch synthase (Fig. 6.6) was essentially the same for all treatments as changes were within the limits of the error. Again, activity in Broom was lower than in Fenman.

In the second experiment, there was an increase in endosperm dry weight over the culture period for the untreated and phosphate treated ears of both cultivars (Fig. 6.7). Phosphate-treated ears had similar dry weights to untreated ears. In Broom, the PEG treated ears showed no increase in dry weight over the culture period, but in Fenman there was a decrease compared to the dry weight at the start of the experiment. Fresh weight was higher than at the start of the experiment in the untreated and phosphate treated ears, but was considerably reduced in the PEG treated ears. Weights were higher for Fenman than Broom from the untreated and phosphate-treated ears but very similar from the PEG-treated ears.

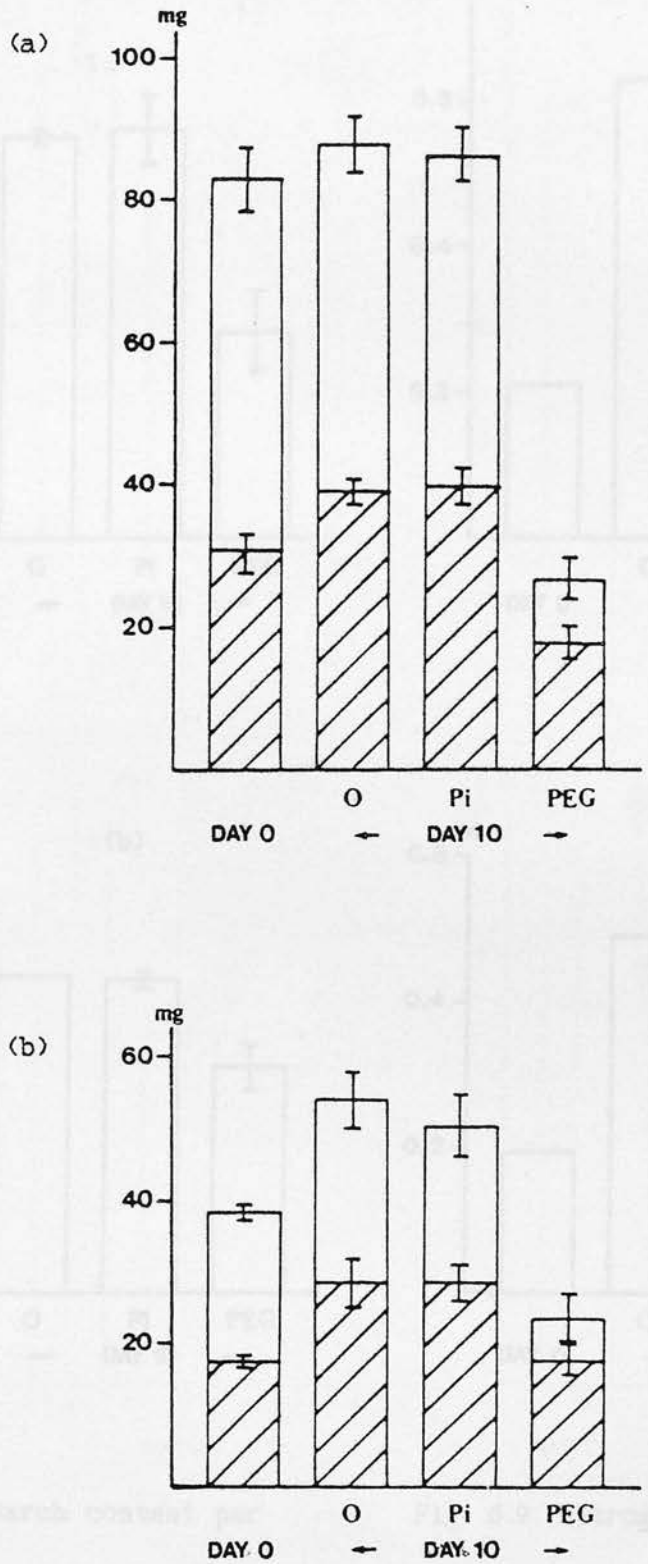
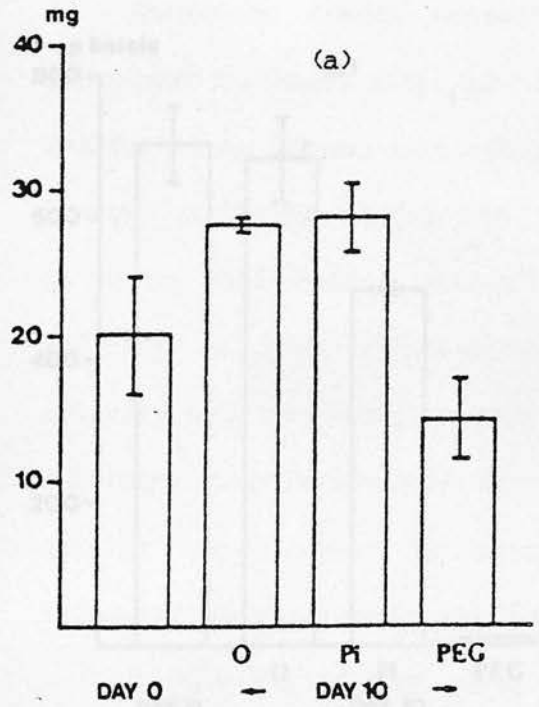
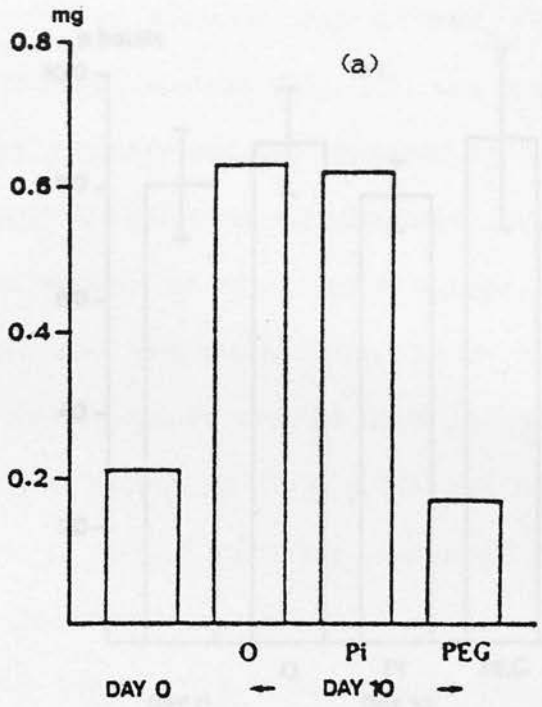
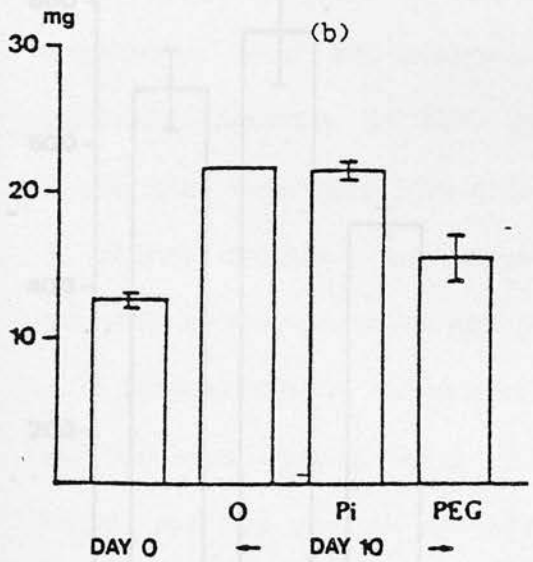


Fig. 6.7. Endosperm fresh weights (whole bars) and dry weights (shaded regions) of (a) Fenman and (b) Broom in the second detached ear culture experiment.







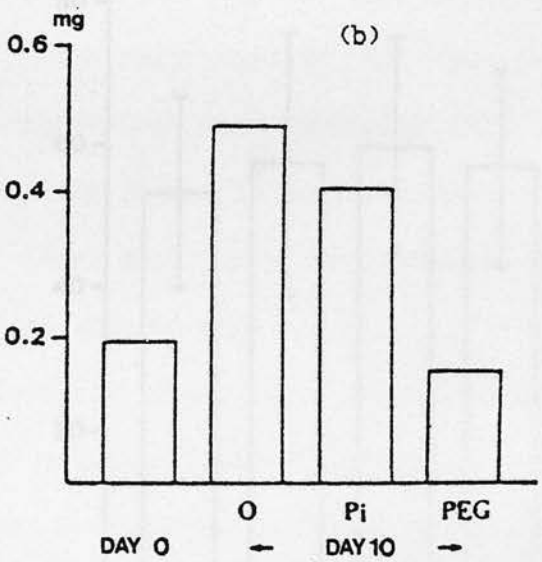
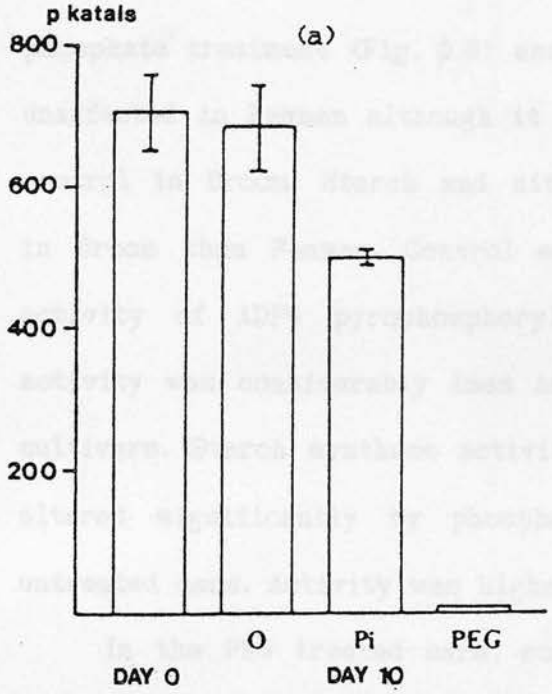


Fig. 6.8. Starch content per endosperm of (a) Fenman and (b) Broom in the second ear culture experiment.

Fig. 6.9. Nitrogen content per endosperm of (a) Fenman and (b) Broom in the second ear culture experiment.



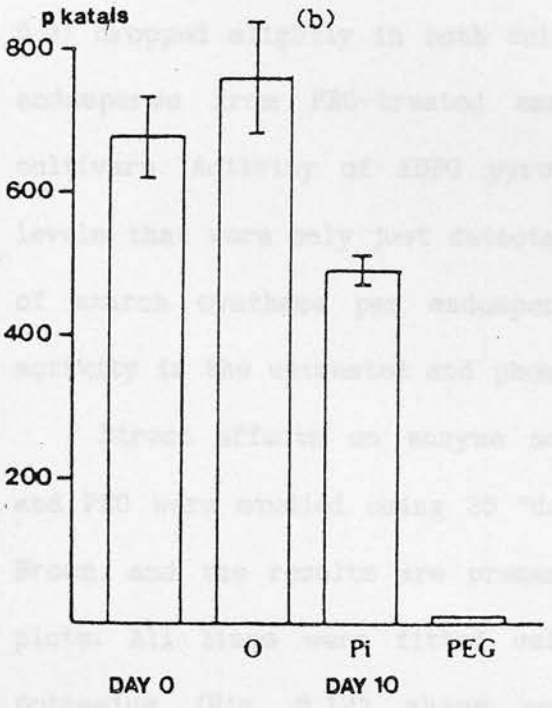
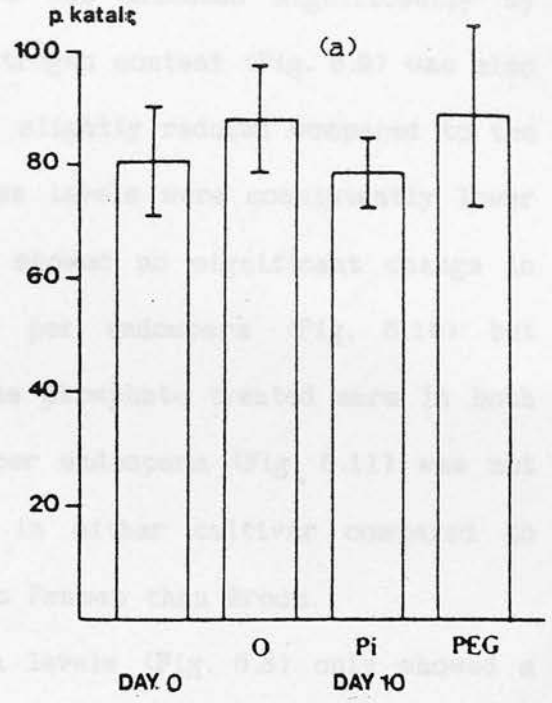


Fig. 6.10. ADPG pyrophosphorylase activity per endosperm of (a) Fenman and (b) Broom in the second ear culture experiment.



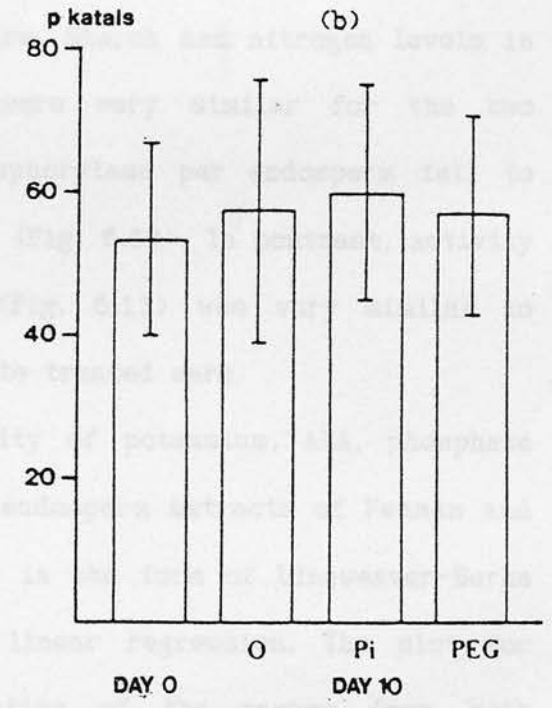


Fig. 6.11. Starch synthase activity per endosperm of (a) Fenman and (b) Broom in the second ear culture experiment.

Endosperm starch contents were not affected significantly by phosphate treatment (Fig. 6.8) and nitrogen content (Fig. 6.9) was also unaffected in Fenman although it was slightly reduced compared to the control in Broom. Starch and nitrogen levels were consistently lower in Broom than Fenman. Control ears showed no significant change in activity of ADPG pyrophosphorylase per endosperm (Fig. 6.10) but activity was considerably less in the phosphate treated ears in both cultivars. Starch synthase activity per endosperm (Fig. 6.11) was not altered significantly by phosphate in either cultivar compared to untreated ears. Activity was higher in Fenman than Broom.

In the PEG treated ears, starch levels (Fig. 6.8) only showed a very slight increase over the starting levels in Broom, and in Fenman showed a decrease compared to the initial level. Nitrogen content (Fig. 6.9) dropped slightly in both cultivars. Starch and nitrogen levels in endosperms from PEG-treated ears were very similar for the two cultivars. Activity of ADPG pyrophosphorylase per endosperm fell to levels that were only just detectable (Fig. 6.10). In contrast, activity of starch synthase per endosperm (Fig. 6.11) was very similar to activity in the untreated and phosphate treated ears.

Direct effects on enzyme activity of potassium, ABA, phosphate and PEG were studied using 25 "day" endosperm extracts of Fenman and Broom, and the results are presented in the form of Lineweaver-Burke plots. All lines were fitted using linear regression. The plot for potassium (Fig. 6.12) shows activation of the enzyme from both cultivars with increasing potassium levels. The kinetic data derived from these plots are given in Table 6.1. In both cultivars, Vmax increased with potassium concentration, but Km changed little although it did fluctuate. Both parameters were higher for Fenman than Broom.

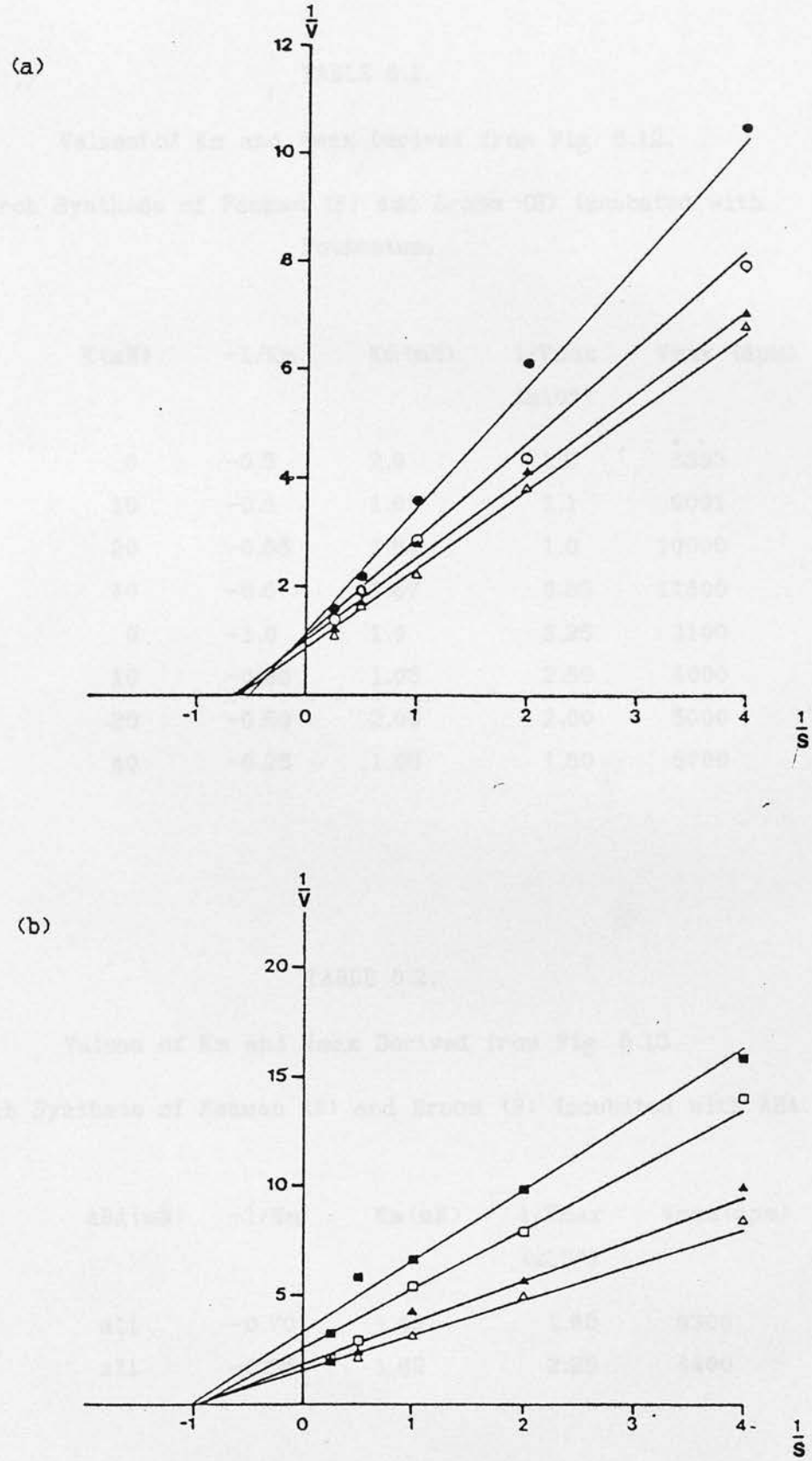


Fig. 6.12. Lineweaver-Burke plot of starch synthase activity at from (a) Fenman and (b) Broom in the presence of 0mM (●,■), 5mM (○,□), 10mM (▲) and 20mM (△) potassium sulphate.

TABLE 6.1.

Values of Km and Vmax Derived from Fig. 6.12.

Starch Synthase of Fenman (F) and Broom (B) Incubated with Potassium.

cv.	K(mM)	-1/Km	Km(mM)	1/Vmax ($x10^4$)	Vmax (dpm)
F	0	-0.5	2.0	1.2	8333
F	10	-0.6	1.67	1.1	9091
F	20	-0.65	1.54	1.0	10000
F	40	-0.6	1.67	0.85	11800
B	0	-1.0	1.0	3.25	3100
B	10	-0.95	1.05	2.50	4000
B	20	-0.50	2.00	2.00	5000
B	40	-0.95	1.05	1.50	6700

TABLE 6.2.

Values of Km and Vmax Derived from Fig. 6.13.

Starch Synthase of Fenman (F) and Broom (B) Incubated with ABA.

cv.	ABA(mM)	-1/Km	Km(mM)	1/Vmax ($x10^4$)	Vmax(dpm)
F	all	-0.70	1.43	1.60	6300
B	all	-0.55	1.82	2.25	4400

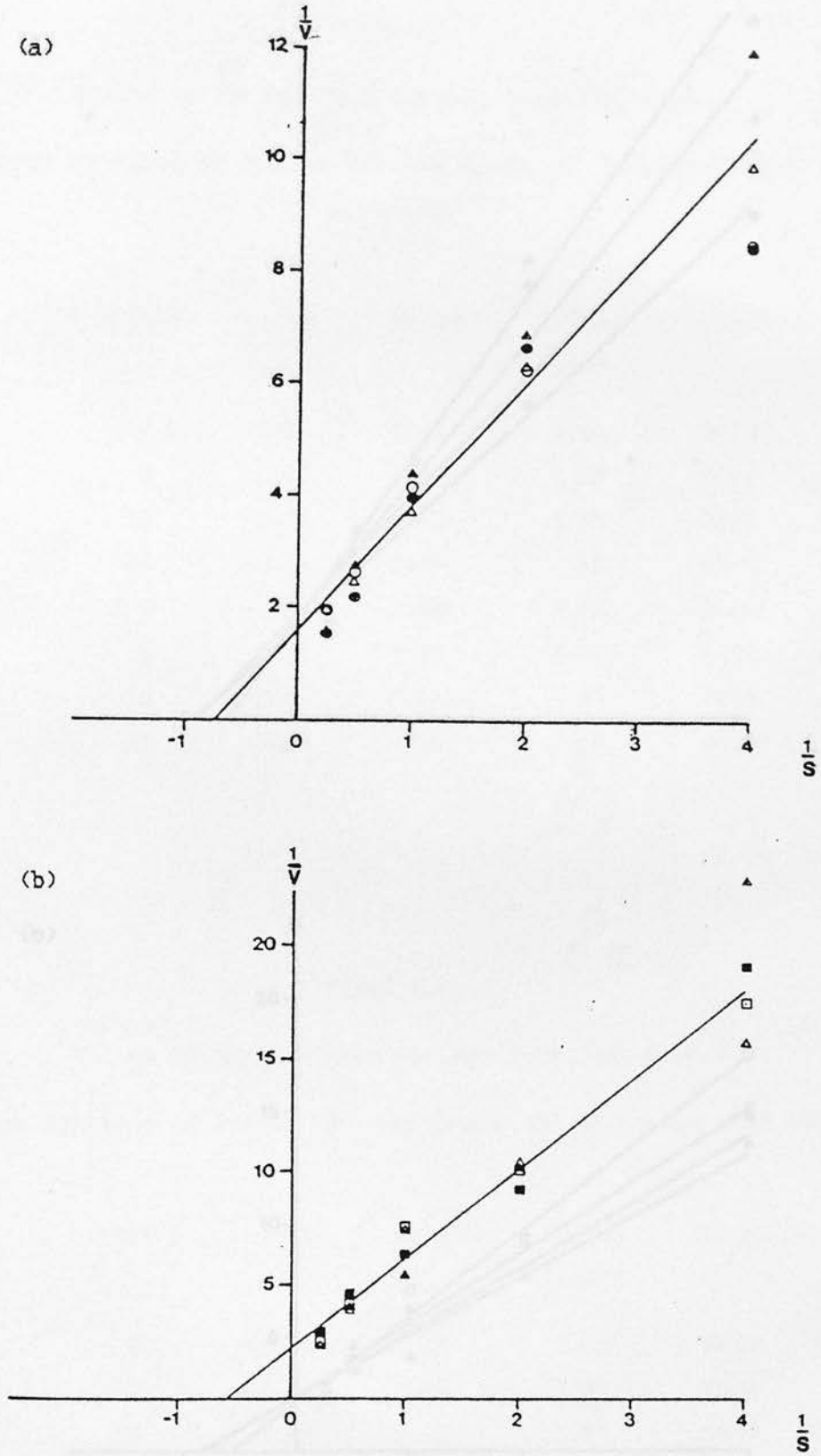


Fig. 6.13. Lineweaver- Burke plot of starch synthase activity from (a) Fenman and (b) Broom in the presence of 0μM (●,■), 2μM (○,□), 4μM (▲) and 10μM (△) abscisic acid.

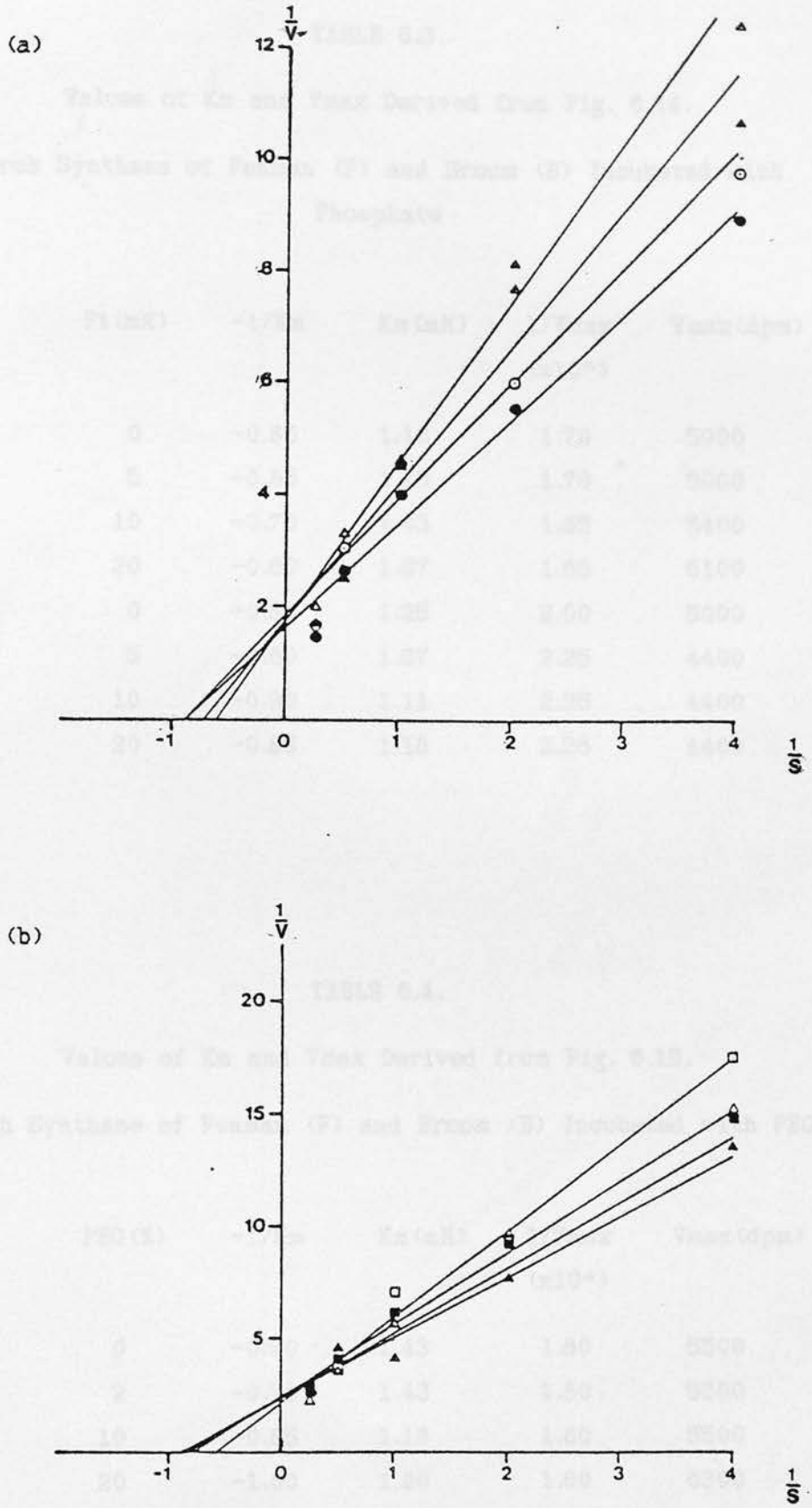


Fig. 6.14. Lineweaver-Burke plot of starch synthase activity from (a) Fenman and (b) Broom in the presence of 0mM (●,■), 5mM (○,□), 10mM (▲) and 20mM (△) orthophosphoric acid.

TABLE 6.3.

Values of Km and Vmax Derived fron Fig. 6.14.

Starch Synthase of Fenman (F) and Broom (B) Incubated with Phosphate

cv.	Pi(mM)	-1/Km	Km(mM)	1/Vmax ($x10^4$)	Vmax(dpm)
F	0	-0.85	1.18	1.70	5900
F	5	-0.85	1.18	1.70	5900
F	10	-0.70	1.43	1.85	5400
F	20	-0.60	1.67	1.65	6100
B	0	-0.80	1.25	2.00	5000
B	5	-0.60	1.67	2.25	4400
B	10	-0.90	1.11	2.25	4400
B	20	-0.85	1.18	2.25	4400

TABLE 6.4.

Values of Km and Vmax Derived from Fig. 6.15.

Starch Synthase of Fenman (F) and Broom (B) Incubated with PEG.

cv.	PEG(%)	-1/Km	Km(mM)	1/Vmax ($x10^4$)	Vmax(dpm)
F	0	-0.70	1.43	1.80	5500
F	2	-0.70	1.43	1.80	5500
F	10	-0.85	1.18	1.80	5500
F	20	-1.00	1.00	1.60	6300
B	0	-0.55	1.82	2.25	4400
B	2	-0.70	1.43	2.50	4000
B	10	-0.95	1.05	2.40	4200
B	20	-1.05	0.95	2.40	4200

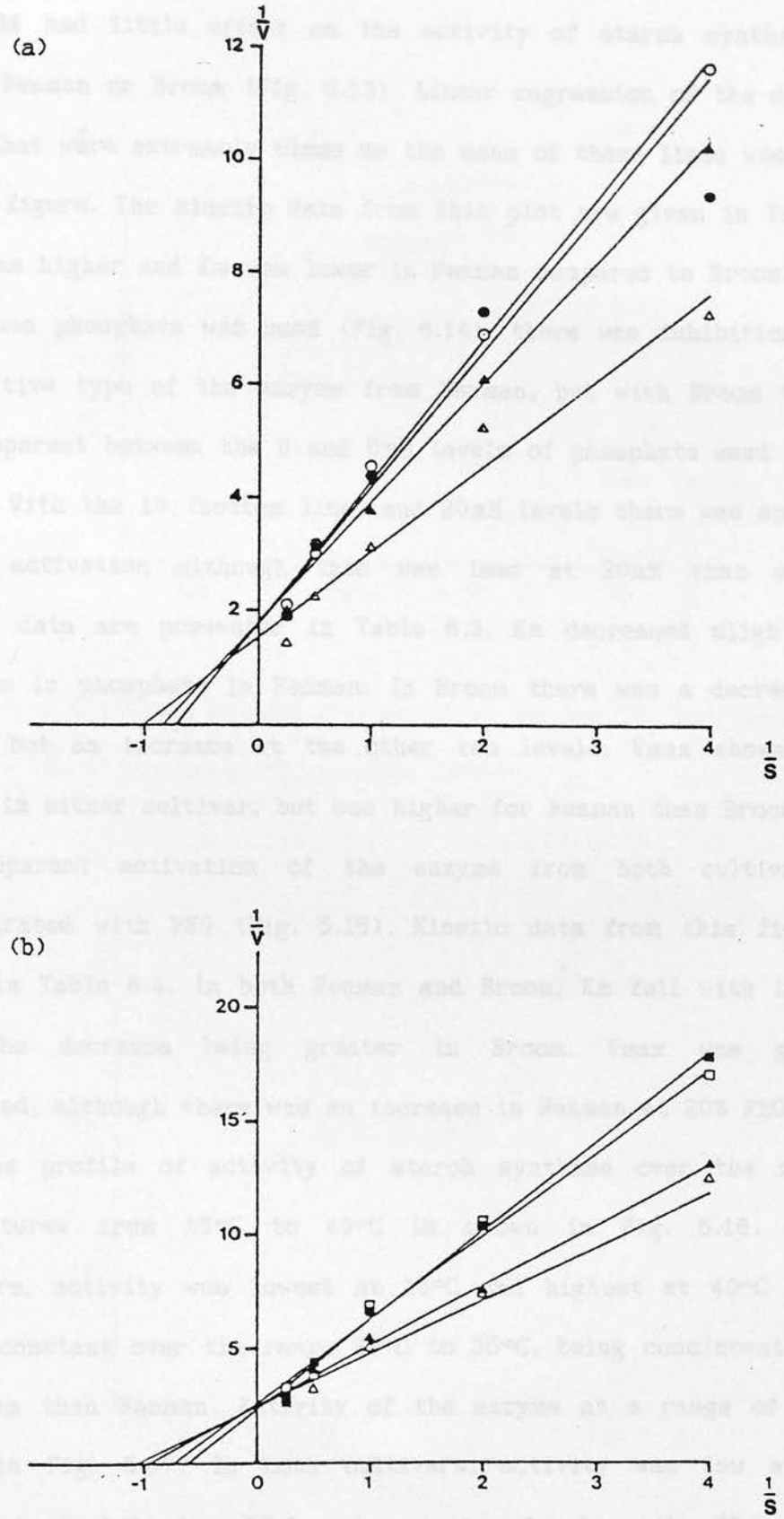


Fig. 6.15. Lineweaver-Burke plot of starch synthase activity from (a) Fenman and (b) Broom in the presence of 0g/l (●,■), 20g/l (○,□), 100g/l (▲) and 200g/l (△) polyethylene glycol 8000.

ABA had little effect on the activity of starch synthase from either Fenman or Broom (Fig. 6.13). Linear regression of the data gave lines that were extremely close so the mean of these lines was plotted in the figure. The kinetic data from this plot are given in Table 6.2. Vmax was higher and Km was lower in Fenman compared to Broom.

When phosphate was used (Fig. 6.14), there was inhibition of the competitive type of the enzyme from Fenman, but with Broom this was only apparent between the 0 and 5mM levels of phosphate used (top two lines). With the 10 (bottom line) and 20mM levels there was apparently slight activation although this was less at 20mM than at 10mM. Kinetic data are presented in Table 6.3. Km decreased slightly with increase in phosphate in Fenman. In Broom there was a decrease from 0-5mM, but an increase at the other two levels. Vmax showed little change in either cultivar, but was higher for Fenman than Broom.

Apparent activation of the enzyme from both cultivars was demonstrated with PEG (Fig. 5.15). Kinetic data from this figure are shown in Table 6.4. In both Fenman and Broom, Km fell with levels of PEG, the decrease being greater in Broom. Vmax was generally unchanged, although there was an increase in Fenman at 20% PEG.

The profile of activity of starch synthase over the range of temperatures from 15°C to 40°C is shown in Fig. 6.16. In both cultivars, activity was lowest at 15°C and highest at 40°C and was fairly constant over the range 20°C to 35°C, being consistently lower in Broom than Fenman. Activity of the enzyme at a range of pH's is given in Fig. 6.17. In both cultivars, activity was low at pH6.5, increased slightly by pH7.0 and was then level until pH8.0. It then peaked at pH8.3 before declining steadily to pH9.0. All these changes in activity were small. Activity was always lower in Broom than in Fenman.

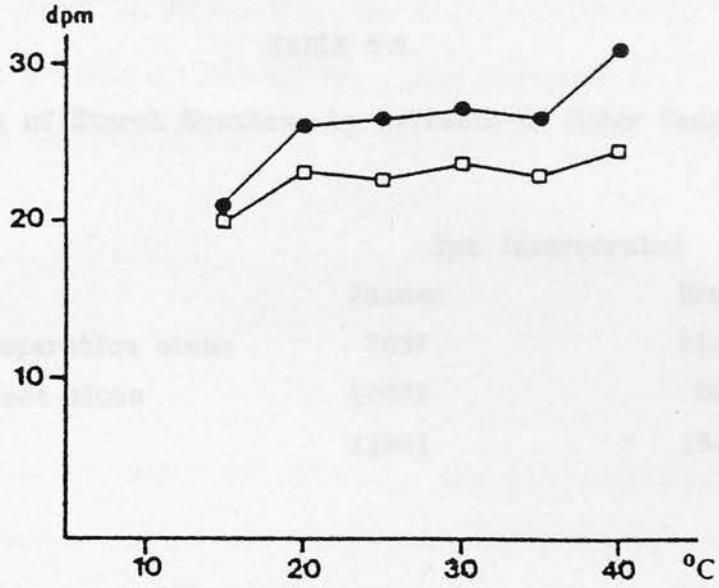


Fig. 6.16. Activity of starch synthase from Fenman (●) and Broom (□) over the temperature range 15-40°C.

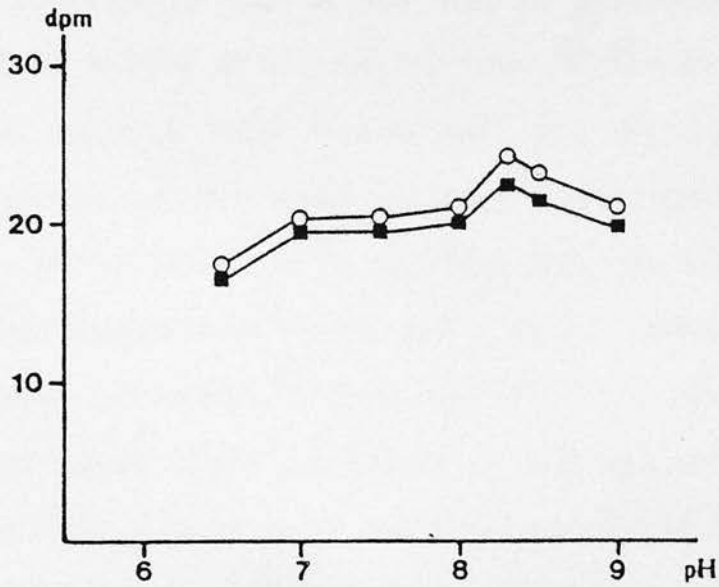


Fig. 6.17. Activity of starch synthase from Fenman (○) and Broom (■) over the pH range 6.5-9.0.

TABLE 6.5.

Inhibition of Starch Synthase by Extracts of Older Endosperms.

	dpm Incorporated	
	Fenman	Broom
Enzyme preparation alone	7037	11481
"Old" extract alone	10062	5926
Combined	13951	15432

"Old" extracts were prepared by homogenising 10 endosperms at 40 "days" after anthesis in 1.5ml 50mM MOPS buffer, pH 7.0 and were filtered through muslin before use. Two "old" extracts were prepared for each cultivar and each was assayed in duplicate for inhibition of 25 "day" starch synthase preparations.

The results of adding extracts of older endosperms to the assay system are given in Table 6.5. The combined activity of the enzyme preparation and the extract of the older endosperms was less than the total of the separate activities of the two in both Fenman and Broom.

6.4 Discussion.

The results from the previous chapter suggested that starch synthase activity may be a rate-limiting factor in the accumulation of starch. Inhibition of this activity could be crucial in the termination of grain growth. Here, the kinetics of that activity are examined and the effects of various substances upon starch synthesis are considered.

The kinetics of starch synthase activity from Fenman and Broom when not influenced by any of the compounds being tested can be investigated by looking at the results from each experiment when the level of the compound being studied was zero. The figures for the kinetic parameters Km (for ADPG) and Vmax vary slightly between the experiments. If the means for Km and Vmax from the four experiments are taken, the enzyme from Fenman has a Km of 1.56mM (0.09) and a Vmax of 6500dpm per endosperm in 15 min (600). The figures for Broom were a Km of 1.65mM (0.13) and a Vmax of 4200 dpm per endosperm in 15 min (400). The numbers in brackets are the standard error of the mean. Vmax gives the maximum possible rate at which starch synthase is capable of synthesising starch. The higher Vmax for the enzyme from Fenman is consistent with the higher rate of starch deposition observed in this cultivar compared to Broom. The value of the Km of starch synthase for ADPG from the cultivars are very similar and are

the same when the limits of the errors are considered. This indicates that the same concentration of ADPG is capable of giving half maximum velocity in both cultivars. That the maximum velocity is lower for the enzyme from Broom suggests that it is limited by a factor or factors other than ADPG concentration. The Km's recorded here are very similar to those found by Cardini and Frydman (1966) for bound starch synthase of beans and peas.

Potassium.

Consistent with previous reports (Nitsos and Evans, 1969), potassium was found to be an activator of starch synthase. The activation gave a Lineweaver-Burke plot which resembled non-competitive inhibition in reverse. This implies that the activator binds to a part of the enzyme molecule other than those sites used by the substrates and in some unknown manner increases the rate at which the reaction proceeds. One possibility is that the activator alters the conformation of the enzyme such that the binding site for a substrate becomes more exposed and therefore binds the substrate more efficiently. A similar type of mechanism has been demonstrated with enzyme inhibitors (Fersht, 1977). As was found for sweetcorn starch synthase (Nitsos and Evans, 1969), the results show a Km for ADPG which was unchanged in the presence of potassium, so it cannot be the binding of ADPG that is affected, but it is possible that is the binding of starch at the active site that is improved. Murata and Akazawa (1968) suggested that potassium caused stronger binding of starch synthase from sweet potato roots to the starch granules which stabilized the enzyme. The increases in Vmax were proportional to the amount of potassium added in both cultivars. Increases were greater in Broom than Fenman as a percentage of the untreated Vmax. Possibly the

lower Vmax for Broom compared to Fenman when untreated means that there is more potential for activation.

In the cultured ears, those grown on potassium-containing media had slightly lower endosperm dry weights than the control ears but this was not due to a reduction in starch or nitrogen content. In Fenman endosperms, higher nitrogen levels were found which suggest that protein synthesis may have been stimulated. Enhancement of wheat grain protein synthesis by potassium through increased amino acid translocation into the grain has been found (Mengel, Secer and Koch, 1981).

Activity of ADPG pyrophosphorylase was slightly lower per endosperm compared to the control. This could be due to reduced specific activity or protein levels of the enzyme. That the lower activity of this enzyme had no effect on the starch content of the endosperm suggests that it is not rate-limiting. However, its activity *in vivo* may not be as high as was measured here. The activity of ADPG pyrophosphorylase recorded was sufficient to yield far more ADPG than could be used by starch synthase. Unlike the *in vitro* study, starch synthase activity per endosperm was unchanged.

The results of the culture experiment do not support the established role of potassium as an activator of starch synthase. The culture medium already contained some potassium and the effects studied were those of adding more in the form of potassium sulphate. It could be that the potassium levels already in the medium are sufficient to saturate requirements and the added potassium then has little effect. The effects seen might be a combination of the effects of potassium and sulphate. The results suggest that sulphate does not inhibit activation by potassium of starch synthase or this would have been observed when potassium sulphate was added to the assay system,

although slightly less activation with potassium sulphate compared to potassium chloride was noted by Nitsos and Evans (1969). It is also possible that dilution of the potassium in the ear tissues or regulation of its uptake gives levels insufficient to activate starch synthase.

Thus, the experiments with potassium showed activation of starch synthase *in vitro*, but in the detached ears the only effects appeared to be slight inhibition of ADPG pyrophosphorylase and an increase in endosperm nitrogen content in one cultivar.

Abscisic Acid.

Inclusion of abscisic acid in the assay system had no effect on the kinetics of starch synthase activity *in vitro*. The level of ABA used was based on that used by Dunwell (1981) to study the effect of ABA on germination of barley grains. King (1979) has shown that ABA can be taken up from the medium by detached ears. In the cultured ears, it gave little change in Broom, but in Fenman endosperm dry weight was slightly reduced although starch and nitrogen levels showed a small increase over the control endosperms. It has been suggested by some workers (see King, 1982) that ABA may act at the RNA level as an inhibitor of RNA translation into protein, although this requires confirmation. Were ABA to act as an inhibitor of RNA translation then this would account for the absence of any direct effect on starch synthase in the assay. However, the higher nitrogen levels found in Fenman suggest that there is more protein present rather than less.

If ABA acts as a non-specific inhibitor of translation and starch synthase is not dependent on renewed synthesis and protein turnover for maintenance of activity, this could explain why starch synthase

activity continues after termination. After ABA inhibition of translation, previously synthesised starch synthase protein would still be present, whereas enzymes requiring continual synthesis would gradually decline in activity as protein was degraded but not replaced. The same presumably also applies to ADPG pyrophosphorylase, as this also was unaffected by ABA in culture.

Phosphate.

With phosphate in the assay system, competitive inhibition was demonstrated clearly by the starch synthase from Fenman. The Vmax remained the same, but the concentration of ADPG required to achieve this increased, as evidenced by an increasing Km. This type of inhibition indicates that phosphate bound to the enzyme in such a way that it inhibited the binding of ADPG. Thus, it appears that one of the binding sites for ADPG to starch synthase is via one of its phosphate groups. The results for the enzyme from Broom were somewhat confused. Inhibition was demonstrated at the 5mM level and at the 20mM level compared to the 10mM level, but the results for the 10mM and 20mM levels showed slight activation over the zero level. All the effects seen with Broom were slight.

In the cultured ears, dry weight, starch and nitrogen content were all unaffected by phosphate, except for a small reduction in the nitrogen content of Broom endosperms. As has been found previously with wheat endosperm ADPG pyrophosphorylase (Riffkin, personal communication), phosphate inhibited activity. The reduction in activity may be the result of lower specific activity and / or amount of enzyme protein. Since Riffkin (personal communication) demonstrated inhibition *in vitro*, a reduced specific activity appears likely. Again, the reduction in pyrophosphorylase activity is not accompanied by a

reduction in starch content supporting the possibility that it has a greater potential activity than is required by the starch synthesis pathway which is limited by other factors. Starch synthase activity was unchanged by the presence of phosphate in this experiment, despite the inhibition observed in the assay system. It is possible that it is less sensitive to phosphate than the pyrophosphorylase and the dilution of the phosphate in the tissues reduced the concentration to levels insufficient to affect starch synthase. This correlates with the results of Rijven and Gifford (1983) who found no effect on starch synthase activity when they incubated wheat endosperm slices in a phosphate-containing medium.

Polyethylene Glycol.

The inclusion of PEG in the starch synthase assay gave results indicating activation. This appears to be an increased affinity for ADPG, as evidenced by the decrease in Km of the enzyme from both cultivars with increasing levels of PEG. Maximum velocity was unchanged but the level of ADPG required to achieve this was lower. It is not clear how increased affinity could occur. One possibility is that the presence of PEG or the lower water levels caused by its presence altered the conformation of the enzyme such that the active site became more exposed, facilitating binding with ADPG.

When PEG was included in the culture medium, endosperm dry weight showed no increase over the 10 day culture period in Broom and it dropped in Fenman. In Broom, this was caused by compensation of the slight starch increase with a drop in nitrogen levels. In Fenman the result was due to reduction in both starch and nitrogen content. It is possible that the drop in water content over the culture period, shown by the fact that endosperm fresh weight is not much higher than

the dry weight, has led to some mobilization of reserves in an attempt to maintain the viability of the grain over a period of apparent drought. It certainly appears that most if not all the synthetic mechanisms are non-operative. In the case of starch synthesis, this can be attributed at least in part to the total lack of ADPG pyrophosphorylase activity. In contrast, activity of starch synthase per endosperm was unaffected by the inclusion of PEG. The maintained activity of starch synthase in this experiment is consistent with its maintained activity after the onset of water loss in the intact plant that was observed in Chapter 5.

Other Factors.

The results of these experiments, and particularly those with PEG, suggest that starch synthase is a fairly stable enzyme. This was also demonstrated in the pH and temperature profiles, in which there was relatively little variation over the ranges tested. Many enzyme systems are inactivated by temperatures of over 37°C, but at 40°C the activity of starch synthase was, if anything, higher than at the lower temperatures.

The results of the experiment in which extracts of older endosperms were added to the 25 day enzyme preparation do suggest that there is some factor in the older extracts which is causing inhibition of starch synthase activity, and which could therefore inhibit activity *in vivo*. This factor presumably is lost or inactivated during the enzyme preparation process.

Starch synthase appears to be the rate-limiting step in starch deposition (Chapter 5), although previous results suggest that it is not responsible for termination. However, the results presented here suggest that there may be an inhibitor operating *in vivo* which is not

present or operative *in vitro*. It is also possible that a drop in ADPG pyrophosphorylase activity could cause termination by reduced ADPG supply, although such a reduction would have to be very large to limit starch synthase activity. The PEG experiment showed how drastic the effect on starch synthesis could be when this enzyme was totally inactivated in response to reduced water levels.

7. CONCLUSIONS.

The results presented in this thesis serve to demonstrate that the process of termination of dry matter accumulation in a developing wheat grain is extremely complex. Various possible causes of termination have been investigated.

Water loss has been associated with the termination of dry matter deposition on many occasions raising the possibility that onset of water loss is the trigger. Attempts have been made in the present work to determine whether or not onset of water loss or termination of dry weight and starch accumulation occurred first. In the majority of cases water loss and cessation of dry matter accumulation were observed to occur at the same time, even when measurements were made at close intervals. However, in one or two cases, there was an indication that onset of water loss occurs first. The necessarily destructive nature of the methods used prevents consecutive measurements of the same grains and the errors encountered between different grains, although small, were often of a similar size to the changes which occurred over the periods between measurements, particularly when these were close together. A possible way to overcome this might be by measurements with very large numbers of grains, hoping to reduce the errors to very low levels.

Evidence in favour of the theory that onset of water loss triggers cessation of dry matter accumulation is given by the experiments with PEG. Inclusion of PEG in the culture medium resulted in water loss from detached ears. Starch deposition also ceased, despite apparent activation *in vitro* of starch synthase by PEG or the low water environment it induced. The conclusion that low water

content causes starch deposition to stop must be treated with some caution since a direct effect of PEG cannot be ruled out. Evidence that onset of water loss does not trigger cessation of dry matter accumulation was found in the experiments using caryopsis culture. The cultured caryopses failed to lose water in the usual fashion but starch deposition did not continue.

Another possible cause of termination is the loss of respiratory activity. This was demonstrated to occur at the same time as or slightly earlier than the onset of water loss and before cessation of starch deposition. Loss of energy-producing pathways could result in the inactivation of synthetic pathways and also of mechanisms of uptake into the grain. However, water efflux from the grain is also thought to be an active process requiring metabolic activity. A possible explanation is that the cells thought to be active in removing water from the grain are the xylem parenchyma cells which are located within the vascular bundle of the crease in the pericarp green layer. It may be that metabolic activity in this layer might be sustained longer than in the endosperm. Oxygen exchange measurements indicated that there was some respiratory activity in the caryopsis until maturity, although this cannot be identified as being in any particular tissue. The theory that respiratory activity might be an important factor in the termination process also raises questions about the role of pericarp photosynthetic activity. This starts to decline some time earlier than respiratory activity, apparently due to a reduction in chlorophyll levels. The cause of chlorophyll breakdown is not known. These processes may form links in a chain of programmed events leading to termination. If this is the case, then it will be exceedingly difficult to identify the "cause" of termination.

About 70% of the final endosperm dry weight is starch. Accumulation of starch was shown to cease at the same point as dry matter deposition and this was not the result of lower sucrose levels in the endosperm. The possibility that cessation of starch deposition is the result of reduced enzyme activities was then investigated.

The direct control of starch deposition was shown to be closely related to the activity of the enzymes ADPG pyrophosphorylase and starch synthase. The granule-bound form of starch synthase was shown to be the most active in starch synthesis and the results suggest that this enzyme is important in determining the rate of starch deposition. Starch synthase activity was highest in the cultivar with the highest rate of starch deposition, and starch deposition in this cultivar (Fenman) was affected to a greater extent by the high temperature stress, which was reflected in the greater reduction in starch synthase activity. It was also found that the Vmax for Fenman was higher than that for Broom, correlating with the higher rate of starch deposition observed. Of course, these effects may be found with many other enzymes of the starch synthesis pathway which were not investigated here. The fact that rates of starch synthesis which can be calculated to be possible from the activities of starch synthase recorded are very similar to those which were actually found suggests that starch synthase is the rate-limiting step in the pathway. It is a characteristic of rate-limiting enzymes that their maximum rate of reaction is equal to or only slightly higher than the observed rates of product synthesis.

ADPG pyrophosphorylase has been implicated in the regulation of starch synthesis in leaves. However, its role in the regulation of starch synthesis in storage organs is unclear. In the present work, it was found that the ADPG pyrophosphorylase present was capable of

producing far more ADPG than could be used by starch synthase. Since ADPG does not accumulate in the immature wheat endosperm the implication is that the enzyme does not operate at maximum rates *in vivo*. The results showing that pyrophosphorylase activity was reduced by potassium (or sulphate) and phosphate without reducing starch accumulation supports the hypothesis that ADPG pyrophosphorylase may not be an important site of regulation of the rate of starch synthesis in reserve tissues. However, this does not mean that this is not the site that controls the termination of starch deposition. Indeed, in the experiment with PEG, one of the factors apparently contributing to the failure to synthesise starch was a complete lack of ADPG pyrophosphorylase activity. Although starch synthase appears to be rate-limiting during grain-filling, the high sensitivity of ADPG pyrophosphorylase to lack of water may make it the causal factor for termination of starch deposition. Starch synthase activity was maintained until well after termination of starch deposition and was not limited by low water levels. However, the results indicate that extracts of older endosperms contain an inhibitor of starch synthase activity which may be important in termination. These conclusions assume that both enzymes are present and active within the amyloplast which has not been shown. For starch synthesis to occur, ADPG pyrophosphorylase must be present to supply ADPG to the granule-bound starch synthase, and in turn must be supplied with glucose-1-phosphate.

It is possible that the events described here are all part of a pre-determined path. From the results presented in this thesis, the first event in such a sequence is the decline in photosynthetic activity and chlorophyll content of the pericarp. Decline in chlorophyll levels is also the first visible sign of approaching

maturation. It is followed by decline in respiratory activity and in water content, which occur at almost the same point. Finally comes the termination of dry matter deposition and starch accumulation, which together are defined as termination of grain growth. This may be the result of inhibition of starch synthase activity and / or reduced ADPG pyrophosphorylase activity. Since it appears that deposition of nitrogen-containing compounds may continue slightly after this point and there is still slight respiratory activity, it is clear that not all grain processes cease at the time of termination of dry matter and starch deposition. The main conclusion from the work described here is therefore that the termination of grain growth is the result of a programmed sequence of events which may have its origins before or very early in grain development.

Various possibilities for further study have been raised by this work. One of the most important in relation to termination of growth is to identify the inhibitor of starch synthase activity, since this factor may be critical in determining when termination of starch deposition and growth occurs. It is also necessary to establish where and when this factor is synthesised and whether it is stored or modified before becoming operational. Its specificity or otherwise for starch synthase should be determined. The possibility that the activity of starch synthesising enzymes is maintained by continued synthesis and turnover needs examining. If this is occurring, termination might be due, at least in part, to reduced enzyme synthesis without a decrease in turnover. The effect of plant growth regulators on the various processes affecting termination of grain growth also requires further investigation. Possibly further development of caryopsis culture would assist with this, as a convenient way of administering sustances such as plant growth

regulators to the developing caryopsis without intervention of other plant parts. Finally, the inter-dependence of the various processes connected with termination needs to be established.

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